THE EFFECT OF INTERVAL BETWEEN INSEMINATIONS AND SEMEN HANDLING ON IN-VIVO AND IN-VITRO FERTILITY OF FROZEN-THAWED BOAR SPERM

BY

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THESIS

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ABSTRACT

Cryopreserved gametes have played a substantial part in the advancement of genetic progress in the production livestock industries. However, the swine industry has not yet been able to capitalize on the potential of frozen-thawed boar sperm (FTS), due to the biological sensitivity of the sperm cells and the lower resulting pregnancy rates and litter sizes. This thesis will outline the current swine reproductive production practices, provide a brief history of boar sperm cryopreservation as well as the advancements in cryopreservation technology and evaluation. This will also report on the impact of artificial insemination in the swine industry with insight into the physiological response to AI, research using frozen-thawed boar sperm, and proper insemination timing relative to ovulation.

Experiment one of this thesis determined the *in vitro* lifespan of frozen-thawed boar sperm to be used for artificial insemination. Many studies have been performed for determining the *in vivo* lifespan of frozen-thawed boar sperm, however very little is known about its *in vitro* lifespan. Samples (n = 20) were evaluated for motility and membrane integrity at the time of thawing and at 2, 6, 12 and 24 h intervals after thawing. Results indicated FTS can be diluted and stored at 17 or 26 °C for up to 2 h with no significant decrease in motility and membrane integrity ($P > 0.10$). Motility remained stable for only 2 h following thawing at all storage temperatures, however, storage at higher temperature showed a gradual decrease in post-thaw quality ($P < 0.001$). Additionally, it should be noted that viability was considerably less sensitive to storage temperature and duration, with samples held at 17 °C and 26 °C showing a

marked reduction at 12 and 6 h, respectively. Storage of FTS at 37 °C, reduced motility and membrane integrity within 2 h, and samples were non-motile and non-viable by 24 hours.

Experiment two was performed to determine the effect of interval between inseminations using FTS and its effect on pregnancy rate, litter size, and fetal paternity. We hypothesized that gilts inseminated at greater intervals would have increased pregnancy rates and litter sizes than those inseminated at a closer interval, accounting for variation in time of ovulation and the shortened lifespan of frozen-thawed boar sperm in the female reproductive tract. Gilts (n = 191) were assigned to one of the following treatments 1) AI at a 4 h interval (34 and 38 h); 2) AI at an 8 h interval (30 and 38 h); or 3) AI at a 16 h interval (22 and 38 h). First and second inseminations were from unique sires to allow for paternity identification. Interval between inseminations affected pregnancy rate ($P < 0.001$) with gilts inseminated at 8 and 16 h intervals having a greater pregnancy rate than those inseminated at 4 h intervals. Litter size and embryo survival was not affected by insemination interval ($P > 0.10$). Gilts that ovulated by 36 and 48 h after the onset of estrus had greater pregnancy rates ($P < 0.001$) than those that ovulated by 24 and 60 h after the onset of estrus, but, estrus to ovulation interval had no effect on litter size or embryo survival ($P > 0.10$). Interval between inseminations had no effect on the proportion of fetuses sired by the second AI ($P > 0.10$); however, there was an interaction of interval between inseminations and estrus to ovulation interval ($P < 0.05$). The results of this experiment indicate that when FTS is used in a double AI system, highest fertility occurs when gilts are mated at 8 and 16 h intervals with estrus detection occurring at 12 hour intervals.

The results of experiment one could have implications for handling and processing procedures for *in vivo* and *in vitro* fertility. Increased *in vitro* lifespan could allow for multiple dose preparation and storage prior to insemination. Additionally, the post-thaw quality results

of samples held at 37 °C could be an indicator for *in vivo* post-thaw sperm survival patterns. With experiment two, very little information is available on the optimal insemination interval to account for the short lifespan of FTS, variation in time of ovulation, and an immune response due to artificial insemination. Knowledge from these studies may help improve timing for estrus detection and artificial insemination to optimize timing and increase fertility when using frozen-thawed boar sperm.

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Chapter 1. Current priorities of commercial swine production

Pork accounts for over 40% of the red meat consumed annually worldwide (Gerrits et al., 2005). With global pork production and consumption exceeding 100 million metric tons in 2010, China is currently the global leader in pork production and consumption, with the European Union, the United States, Brazil, Russia, Vietnam, and Canada being other substantial producers (USDA, 2011). It is predicted that by the year 2020, global pork demand will reach approximately 125 million tons, with a majority of the consumption being in developing countries (Gerrits et al., 2005). With this projected growth in pork consumption, swine producers must be able to increase swine production as well as the efficiency of their operations in order to accommodate the growing global demand.

Swine production and management in the United States has experienced significant changes over the past 20 years. Swine production systems, in general, have shifted from high numbers of small family farms with outdoor production, to fewer highly concentrated, indoor confinement systems. From 1992 to 2004, the number of swine operations in the United States decreased by approximately 70% (240,000 to 70,000), while the national swine inventory remained relatively constant (60 million head). This is attributed to the substantial increase in the number of swine operations with greater that 2,000 head, and a substantial decrease in the number of in smaller herds (Key and McBride, 2007). Additionally, swine production has become more specialized, moving away from the traditional farrow-to-finish operations in favor of segmented production systems specializing in a single phase of production such as breeding and gestation, farrowing, nursery, and grow-to-finish (Key and McBride, 2007). With this shift in production trends, producers have been searching for opportunities to increase the efficiency of their operations.

Technological advancements have been adopted to increase feed efficiency, improve swine housing and handling, manage herd health and biosecurity, and accelerate genetic progress through artificial insemination and reproductive management. All of these innovations have helped increase production efficiency, as well as help manage risks for swine producers (Gerrits et al., 2005; Key and McBride, 2007).

Reproductive efficiency is paramount in profitable swine operations, and has been a driver for profitability. One of the most influential advancements for improving reproductive efficiency has been the utilization of artificial insemination. This results from the ability to disseminate superior genetics while minimizing disease transmission (Bailey et al., 2008; Gerrits et al., 2005; Key and McBride, 2007). Artificial insemination (AI) was first commercially available in the 1970s, and while initially underutilized in the United States, by the 1990s artificial insemination with liquid, extended semen had increased exponentially (Gerrits et al., 2005; Roca et al., 2006). By 2006, it was estimated that artificial inseminations with liquid extended semen accounted for over 75% of all swine matings in the United States (Roca et al., 2006). Internationally, swine AI has experienced tremendous growth throughout Europe, South America, and East Asia (Riesenbeck, 2011). Countries with advanced infrastructure and specialized swine production units have embraced the advantages of AI. These include higher reproductive efficiency, the ability to mate multiple females from one ejaculate, the possibility of disseminating superior genetics, and finally, improved herd health and biosecurity (Riesenbeck, 2011).

While liquid extended semen has become the standard for AI use in the swine industry, its major limitation to date is storage longevity to ~7 days. In contrast, frozen-thawed boar sperm could extend used especially in for export, genetic banking, and as a biosecurity measure. For

each, the longevity of storage in a quiescent state is a distinct advantage allowing for variations in storage time ranging from days to years (Bailey et al., 2008; Johnson et al., 2000). However, there have been several limitations preventing widespread utilization of frozen-thawed boar sperm. The main deterrent of frozen thawed boar sperm is the reduction in pregnancy rates (<70%) and litter sizes (~10 pigs/litter) compared to liquid extended semen (Johnson, 1985b; Polge et al., 1970). This decrease in fertility is often attributed to damage that occurs during the cryopreservation process that manifests as disruptions of the sperm plasma membrane, mitochondrial function, as well as the cryocapacitation phenomenon (Bailey et al., 2003; Watson, 2000). Considerable research has been conducted in the field with frozen-thawed boar sperm over the past several decades. This research has lead to several advancements in cryopreservation procedures, optimal insemination methodologies, and most importantly, proper insemination timing relative to ovulation; artificial insemination using frozen-thawed boar sperm has yielded acceptable fertility results (Spencer et al., 2010).

Chapter 2. Cryopreservation of boar sperm

2.1 Brief history of gamete cryopreservation

The ability to preserve gametes has been a topic of research for several centuries, with reports of successful cryopreservation and revival of gametes in many species dating back to the 1700's. However, it was not until 1949 with Polge's discovery of glycerol as a cryoprotectant that the preservation of gametes and somatic cells was a viable procedure (Bwanga, 1991; Johnson et al., 2000; Rath et al., 2009). Successful cryopreservation techniques for bull and boar sperm were developed in the 1950's yielding pregnancies from frozen bull (1951) and frozen boar (1970) sperm using artificial insemination (Bwanga, 1991; Johnson et al., 2000). While the first pregnancies from frozen-thawed boar sperm were obtained in 1970, pregnancy rates and litter sizes were considerably lower than that of liquid, extended semen (Johnson et al., 2000). Though there is evidence of variable *in vivo* fertility among different species, a decrease in fertility following cryopreservation has become the accepted norm and is standard across species, with even the most optimized cryopreservation protocols yielding 40-50% post-thaw motility (Bailey et al., 2008; Watson, 2000). Along with this reduction in motility, many species observe differences in the sperm cell membrane structure, mitochondrial activity, membrane permeability, as well as capacitation status in frozen-thawed cells as compared to fresh sperm cells (Bwanga, 1991; Colenbrander et al., 2000; Cremades et al., 2005; Holt, 2000b). Significant research has been conducted on several areas of the cryopreservation procedure in an attempt to increase post-thaw quality and ultimate fertility. Experimentation has included variations in freezing procedures and cryoprotectants (Fiser et al., 1993; Hernández et al., 2007c), freezing rate (Hernández et al., 2007c; Johnson et al., 2000; Thurston et al., 2003), and finally thawing

rate and conditions (Fiser et al., 1993; Garcia et al., 2010). With continued advancement in cryopreservation and post-thaw semen handling protocols, there may be potential to increase the post-thaw quality of frozen-thawed boar sperm.

2.2 Cryopreservation methodology and media

Cryopreservation of sperm is a multi-step protocol divided into two phases, a cooling phase and freezing phase (Almlid et al., 1987). Beginning at collection, the ejaculate must be extended and cooled to 17 °C, which allows for equilibration of the sperm in seminal plasma and extender. This allows the sperm's plasma membrane to interact with seminal plasma proteins that protect the cell from damage occurring during freezing and thawing (Pursel and Johnson, 1975). The extended ejaculate is then centrifuged at 800 x *g* for 10 minutes at 17 °C to remove seminal plasma and for concentration of the sperm cells (Almlid et al., 1987; Pursel and Johnson, 1975). The sperm pellet is diluted to the desired concentration in cooling media containing egg yolk, glycerol, and surfactants (Almlid et al., 1987). The sperm are cooled to 5 °C and held for over 2 h to allow for membrane equilibration prior to freezing. The freezing phase of cryopreservation begins with the addition of freezing media, composed of egg yolk and 2 to 3% glycerol, to achieve the desired freezing volume and concentration (Pursel and Johnson, 1975). Finally, the sperm are then packaged, either in straws or plastic bags, and placed into a computer controlled-rate freezing system or over liquid nitrogen vapors to freeze the contents. Following freezing, the samples are stored in liquid nitrogen at -196 °C.

The transition of water from liquid to ice is the major cause of sperm damage during cryopreservation. To circumvent this, specialized freezing media has been developed to protect

sperm cells from damage to the plasma membrane, oxidative stress, and cryoinjury (Rath et al., 2009; Rodriguez-Martinez and Wallgren, 2011). Phospholipids, the basic component of freezing media, protect the plasma membrane during cryopreservation (Maldjian et al., 2005) and egg yolk based extenders are commonly used in boar sperm cryopreservation procedures (Holt, 2000a). Surfactants, such as Ovrus ES Paste, are also included in cryopreservation media to breakdown excess lipids in freezing media that are absorbed by the sperm plasma membrane (Almlid et al., 1987; Holt, 2000a) ultimately improving post-thaw motility, membrane viability, and acrosome integrity. Seminal plasma has also been used as an additive both prior to cryopreservation (Hernández et al., 2007b) and after thawing (Abad et al., 2007b; Garcia et al., 2010; Okazaki et al., 2009b). Experimentation with seminal plasma is based on the theory that specialized proteins bind with spermatozoa and stabilize the sperm's plasma membrane, providing protection against cold shock, preventing premature acrosome reaction, and ultimately aiding in oviductal binding and fertilization (Rath et al., 2009). However, there has been no clearly documented effect on conception and farrowing rate (Okazaki et al., 2009b). Finally, research has indicated the inclusion of antioxidants such as butylated hydroxytoluene, catalase, superoxide dismutase, glutathione, dithiothreitol and α – tocopherol (Holt, 2000a; Peña et al., 2003; Rath et al., 2009; Woelders et al., 1995) prevent oxidative membrane damage that occurs during cryopreservation and helps maintain membrane viability (Hernández et al., 2007d; Rath et al., 2009).

The discovery and introduction of specialized cryoprotectants has revolutionized the freezing process, through protecting cells during phase transitions in the freezing process. Glycerol is commonly used as a cryoprotectant in swine. While cytotoxic at warm temperatures, when glycerol is added to sperm using a stepwise addition at low temperatures (~5°C) the slow rate of

permeation through the plasma membrane allows for the displacement of intracellular water. Additionally, glycerol depresses the freezing point, slowing ice crystal formation, and displaces intracellular water causing ice crystal formation to occur in the media surrounding the cells (Holt, 2000a; Rath et al., 2009; Rodriguez-Martinez and Wallgren, 2011). Other compounds such as dimethyl sulphoxide (DMSO), ethylene glycol, and ethylenediaminetetraacetic acid (EDTA) have been evaluated as viable alternatives for cryoprotectants due to their ability to depress the freezing point of cells by mediating the ion concentration in cells. However, no compounds have been proven as effective as glycerol (Holt, 2000a; Johnson et al., 2000). Though many cells do survive the cryopreservation process even with the addition of specialized freezing media and cryoprotectants, and use of optimized protocols, there is still considerable damage to sperm cells (Rath et al., 2009).

Freezing and thawing rates are considered to be some of the most critical influences on sperm survival during the cryopreservation process. Determination of the optimal cooling, freezing, and thawing rate for boar sperm is paramount to ensure good quality sperm following cryopreservation. Temperature sensitivity of boar sperm begins following collection. The ejaculate is collected, diluted, and slowly cooled in two phases, the first of which takes spermatozoa from 37 °C to 17 °C over 2 h (Holt et al., 1996). Then following the addition of cooling media, samples are cooled from 17 °C to 5 °C over 2 to 4 h (Spencer et al., 2010). The rate of freezing from 5 °C to -196 °C is the most critical steps contributing to damage associated with ice crystal formation and osmotic stress. In some original cryopreservation procedures, boar sperm was frozen over liquid nitrogen vapour at a constant rate of approximately 1°C/min (Almlid et al., 1987). The introduction of controlled-rate programmable freezers have allowed for cryopreservation procedures to evaluated alternative freezing rates (approximately 30-

50°C/min) allowing for proper timing of the release of intracellular water to prevent ice crystal formation within the cell (Hernández et al., 2007c; Rath et al., 2009; Rodriguez-Martinez and Wallgren, 2011). Following the addition of freezing extender, semen is packaged and is then frozen using a multistep freezing curve as follows: 1) from 5 °C to -5 °C (5 °C/minute) allowing for the osmotic equilibration (Bwanga et al., 1991b; Pursel and Park, 1985), 2) a rapid decrease in freezing rate from -5°C to -140 °C/minute to prevent the formation of ice crystals within the cell membrane at lower temperatures (Bwanga et al., 1991b; Eriksson et al., 2002; Garcia et al., 2010) and then 3) cooling from -140 °C to – 196 °C by plunging into liquid nitrogen for long term storage.

Thawing is a reversal of the effects of the withdrawal of intracellular water, cell shrinkage, and influx of ions, yielding a influx of water into the cell which causes membrane disruption (Holt, 2000a). Consequently, a high thawing rate, such as 1,000 to 1,800 °C/min, at high temperatures (50-70°C) is optimal to prevent the reformation of ice crystals (Fiser et al., 1993). However, thawing procedures are highly dependent on the semen packaging system utilized. When cryopreservation procedures were first developed, semen was frozen in ampules or pellets using dry ice (Pursel and Johnson, 1975). As these procedures have been refined, packaging systems have evolved and are now traditionally plastic straws (0.25, 0.5, and 5 mL straws) or plastic bags, known as the MiniFlatPack™ and FlatPack™ (0.5-0.7 mL and 5 mL, respectively) (Bwanga et al., 1991a; Eriksson et al., 2002; Eriksson and Rodriguez-Martinez, 2000; Eriksson et al., 2001; Johnson et al., 2000). In some studies, better post-thaw quality was observed when using lower volume straws and plastic bags (0.25 mL to 0.7 mL), due to the more favorable surface area to volume ratio, allowing for equal freezing and thawing (Bwanga et al., 1991a; Córdova et al., 2002; Pelaez et al., 2006). In contrast to bull sperm which is frozen in 0.25 mL

straws with approximately 10-15 x 10^6 spermatozoa, boar sperm must be frozen at a higher concentration and volume to accommodate the larger numbers of spermatozoa necessary to ensure fertilization. With the need for higher sperm numbers, larger volume straws or plastic bags are often favored for added practicality and convenience. Though these yield lower post-thaw quality, comparable pregnancy rates are observed in field fertility trials and viewed as a more convenient and efficient alternative to several smaller volume straws (Eriksson and Rodriguez-Martinez, 2000; Holt, 2000a).

2.3 Damage associated with cryopreservation

Throughout the cryopreservation process spermatozoa are subjected to many stressors including exposure to low temperatures, oxidative stress, and finally the toxic effects of cryoprotectants (Rath et al., 2009). These challenges alter the cell's molecular structure, compromising sperm function and ability to fertilize oocytes (Rath et al., 2009). Many handling procedures used in the cryopreservation process have been shown to have detrimental effects on semen quality, the most pronounced of which is sperm cooling from 37 °C to 17 °C. Cold shock is the phenomenon attributed to the phospholipid phase transition effects during cooling from 37°C to 17°C (Holt, 2000a; Holt, 2000b; Watson, 2000) which inhibit the cell's ability to regulate fluid and ion movement across the cell membrane (Hernández et al., 2007a). Research by Pursel and Park (1985) has shown that incubation of spermatozoa with seminal plasma helps stabilize the plasma membrane prior to cryopreservation. Additionally highly concentrating sperm helps develop a resistance to cold shock in storage and damage during cryopreservation. Though adaptations in cryopreservation procedures and freezing medias have

been shown to minimize the amount of damage done to sperm cells, there is still irreparable sperm damage affecting function associated with improper cooling (Hammerstedt et al., 1990). As the cooling phase transitions to the freezing phase of cryopreservation, the spermatozoa are subjected to additional challenges such as subzero temperatures and the formation of ice crystals within the cell membrane. Ice crystal formation and changes in osmotic balance during freezing and thawing are the two major causes for sperm cryo-injury, causing disruptions in the sperm plasma membrane lipids and proteins (Watson, 2000). As stated earlier, determination of the optimal freezing and thawing rate is extremely important for ensuring post-thaw quality. Freezing rates that are too slow or too rapid may not allow ice crystal formation outside the sperm cell causing disruption of the cytoplasmic membrane (Bwanga et al., 1991a) which may be compounded during thawing (Holt, 2000a). Changes in osmolarity also cause damage to sperm cells during cryopreservation. Stepwise addition of freezing media is utilized to avoid drastic changes in osmolarity (Hammerstedt et al., 1990; Holt, 2000a). These changes in osmolarity inhibit the ability to regulate the influx of ions and liquid across the sperm plasma membrane (Hernández et al., 2007a). Finally, spermatozoa are extremely sensitive to several cryoprotectants added to traditional freezing medias (Watson, 2000) specifically glycerol, which is known to have cytotoxic effects on sperm (Holt, 2000a; Johnson, 1985b).

In addition to the cryopreservation process, thawing protocols also damage sperm resulting in the loss of selective permeability and structural integrity of the sperm structures essential to maintaining proper function such as the acrosome, head and mid-piece (Rath et al., 2009). This phenomenon, known as "cryocapacitation," accounts for the shortened lifespan and abnormal behavior of frozen-thawed boar sperm *in vitro* and *in vivo* (Holt, 2000a). Cooled sperm behave similar to that of capacitated sperm cells, exhibiting a release of intracellular calcium and

membrane characteristics similar to that of fresh capacitated sperm (Rath et al., 2009; Watson, 2000). Oxidative damage of spermatozoa is common during the freezing and thawing cycles due to the creation of reactive oxygen species (ROS), which in large concentrations can damage cellular molecules such as phospholipids, proteins as well as mitochondrial and nuclear DNA which ultimately leads to cellular apoptosis (Bathgate, 2011; Holt, 2000a; Rath et al., 2009). Other changes related to oxidative damage include increased intracellular calcium concentration, disturbance of cellular mitochondrial function, as well as decreased cellular ATP levels (Rath et al., 2009). To circumvent lipid peroxidation, several cryopreservation medias include chelating agents and antioxidants that scavenge the superoxide anions ultimately converting them to water and oxygen (Bathgate, 2011; Holt, 2000a). One major advancement in the field of frozen-thawed boar sperm research has been the ability to characterize this damage associated with the cryopreservation process and determine its relationship to *in vivo* fertility.

2.4 Methods of sperm evaluation

With increased dependence on artificial insemination, indentifying high fertility boars capable of establishing and maintaining pregnancy has become a priority (Foxcroft et al., 2008; Gadea, 2005). Methodology has been developed to identify infertile or sub-fertile boars, however current protocols are considered ineffective at distinguishing between moderate and highly fertile boars (Foxcroft et al., 2008; Gadea, 2005; Holt, 1995). In recent years, the industry has been searching for a predictive assay as opposed to the retrospective indicators of boar fertility such as conception rates, farrowing rates, and litter size to identify superior boars (Foxcroft et al., 2008). The ultimate goal of semen evaluation is to identify boars that would be capable of producing ejaculates yielding higher fertilization and pregnancy rates (den Daas,

1992) as well as identifying sperm able to survive in the female reproductive tract and fertilize oocytes (Colenbrander et al., 2000). In order establish pregnancy, sperm must be able to pass through cervical mucus, escape phagocytosis in the uterus, enter the utero-tubal junction (UTJ), and form a functional sperm reservoir, where capacitation and acrosome reaction can occur (Colenbrander et al., 2000). Common measures of boar fertility consist of sperm concentration, motility, and morphology, which serve as an indicator of a boar's ability to produce normal, functional sperm (Johnson et al., 2000), however these methods individually offer little value for a prediction of ultimate boar fertility (Gadea, 2005; Sutkeviciene et al., 2009). Potentially, these measures of fertility may be combined with fluorescent staining or *in vitro* fertilization assays to create a multivariate analysis capable of predicting fertility (Gadea, 2005).

Motility is the most widely uses assessment and predictor of spermatozoa survival for both liquid and frozen-thawed boar sperm (den Daas, 1992). The assessment of motility is an important tool in evaluating the ferilitizing ability of the sperm and indirectly the sperm's viability (Foxcroft et al., 2008). Though relatively inexpensive and more predictive of subsequent fertility, sperm motility alone is not an independent predictor of fertility and is a compensable trait, in which larger numbers of sperm can mask apparent lower fertility (Foxcroft et al., 2008). Motility evaluation is traditionally conducted through manual evaluation, with a light or phase contrast microscope; however, advancements have been made in the past several years with the development and incorporation of Computer Assisted Semen Analysis (CASA) systems (Didion, 2008). Using advanced algorithms, CASA systems provide estimates for up to 30 kinematic parameters including progressive, linear motion, sperm velocity, lateral head displacement and other assays that establish sperm viability and capacitation status through fluorescent staining and bicarbonate assays (Rath et al., 2009). Common problems associated

with CASA systems are the high variability attributed to sample preparation and operator error, causing more variation than different systems themselves (Holt, 1995).

Sperm morphology is used as an indicator of the boar's ability to produce high quality sperm capable of fertilizing oocytes (Gadea, 2005). The presence of proximal cytoplasmic droplets and abnormal sperm heads are correlated to infertile males due to either over-collection of boars or testicular damage (Alm et al., 2006; Gadea, 2005). Recent studies have indicated a correlation between normal morphological characteristics, pregnancy rate, and litter size, which are particularly sensitive to normal head morphology (Alm et al., 2006; Gadea, 2005; Tsakmakidis et al., 2010; Xu et al., 1998). Determination of gross morphology is relatively simple through the use of eosin-nigrosin staining, and observation with a phase contrast microscope (Foxcroft et al., 2008).

The freezing and thawing processes are extremely detrimental to sperm quality, and these problems manifest themselves as disruptions in the sperm's plasma membrane structure and function, ultimately yielding lower *in vivo* and *in vitro* sperm quality (Rath et al., 2009). For that reason, protocols have been developed to assess the viability of the plasma membrane, acrosome, and mitochondria as well as capacitation status of frozen-thawed boar sperm cells (Rath et al., 2009). Fluorescent staining was adopted in the 1980's in order to evaluate the functionality and stability of the sperm cell's plasma membrane (Johnson et al., 2000). The combination of SYBR and propidium iodide (PI) have been used effectively as a live/dead stain using both microscopy and flow cytometry in many species including humans, swine, cattle, sheep, rabbits and mice (Almlid et al., 1987; Garner and Johnson, 1995; Johnson et al., 2000; Pelaez et al., 2006). In brief, the ability of the propidium iodide stain to penetrate the sperm's plasma membrane and bind to the nucleolus indicates a compromised sperm cell, while SYBR penetrates all cells with

and binds to DNA (Holt, 2000a). Fluorescent stains such as Hoechst 33258 and ethidium homodimers are also utilized to assess plasma membrane integrity, however, these are often combined with other fluorescent probes (Holt, 2000a; Peña et al., 2007). Recently, attention has been given to utilizing fluorescent staining to determine the functional status of other important cell components. Mitochondrial integrity is evaluated through the use of rhodamine 123, a fluorescent compound, that is permeable to the inner membrane of the mitochondria and binds to actively metabolizing mitochondria (Holt, 2000a). In order the assess the functional state of the acrosome, fluorescent lectins (FITC-PNA) are utilized to determine the proportion of normal acrosome ridges (NAR) (Holt, 2000a). Chlortetracycline (CTC) is often used to determine both acrosome status as well as capacitation status, as it binds to the intracellular calcium that is released during the acrosome reaction and capacitation (Holt, 2000a; Johnson et al., 2000). Though fluorescent stains and other measures of viability often indicate the sperm's viability as well as the ability to maintain homeostasis in the female reproductive tract (Sutkeviciene et al., 2009), they are not an adequate predictor of fertility, as they indicate the membrane and acrosome integrity not normal physiological function (Gadea, 2005; Sutkeviciene et al., 2009).

With the ultimate objective of semen quality assessment being to establish boars capable of yielding acceptable fertilization and pregnancy rates, sperm assays have been developed to identify characteristics and sperm functions vital to fertilization and embryonic development (Foxcroft et al., 2008; Gadea, 2005). Fertilization is a complex multistep process requiring a number of critical events. Within the male, sperm development and seminal plasma components are critical processes, while within the female, sperm transport, capacitation, hyperactivation, and acrosome reaction must occur in order for sperm to penetrate the zona pellucida (Popwell and Flowers, 2004; Rodríguez-Martínez, 2006). It is estimated only 2.5% of sperm are able to

fertilize the oocytes (Holt et al., 2005), and those spermatozoa are a small highly selected sub-population that is not representative of the bulk of the sperm evaluated in the ejaculate (Foxcroft et al., 2008). However, it has become abundantly clear that there is no one comprehensive predictor of *in vivo* and *in vitro* fertility, and that there is substantial variation between boars (Popwell and Flowers, 2004). As such, extensive research has been performed in order to develop an *in vitro* assay that can better predict fertility within the sow by simulating the physiological activity of inseminated boar sperm (Gadea, 2005; Rodríguez-Martínez, 2006). *In vitro* fertilization (IVF) systems that can include IVF (Rodríguez-Martínez, 2006; Xu et al., 1998) sperm-zona pellucida binding assays, oocyte penetration, and competitive fertilization experiments (Braundmeier et al., 2004; Martin and Dziuk, 1977) are all utilized to assess the overall sperm function during the fertilization process, indicating the ability of spermatozoa to interact with the oocytes and explaining the variability in fertilizing ability (Gadea, 2005). While there is limited *in vivo* fertility data to correspond to the *in vitro* fertility assays, individual boar variation is a significant contributing factor for lower fertility (Sellés et al., 2003). Though there is no direct correlation between *in vitro assays* and *in vivo* fertility, these are used to distinguish moderate and highly fertile boars as part multivariate analysis (Gil et al., 2005), incorporating other sperm quality measures including motility, morphology, membrane viability and acrosome status (Braundmeier et al., 2004; Collins et al., 2008; Harkema et al., 2004). With these advancements in evaluation methodology, combined laboratory analysis and assays may be crucial in better predicting frozen-thawed semen capable of fertilization and thereby usable for artificial insemination.

Chapter 3. Artificial insemination in swine

3.1 Transition to artificial insemination in the swine industry

Since the introduction of artificial insemination in the swine industry in the 1970's, many countries throughout Europe, Asia, as well as North and South America have begun capitalizing on the advantages over natural service (Gerrits et al., 2005). It was estimated that in 2000, over 155 million doses of semen were used for artificial insemination in swine and its use has grown exponentially since then, with the widespread use in emerging swine markets through South America and Asia (Riesenbeck, 2011; Weitze, 2000). The primary reason for the implementation of artificial insemination in the swine industry is the ability to increase the rate of genetic progress by allowing for the ability to select males with superior traits, higher reproductive efficiency, decreased labor associated with fewer boars for mating and increased disease control (Roca et al., 2006).

In general, boars selected for use in artificial insemination possess traits that make them valuable to a producer (Robinson and Buhr, 2005). Growth and performance, structural soundness, and carcass traits are considered of high economical value due to the impact on offspring's performance, longevity, and carcass quality (Gerrits et al., 2005; Robinson and Buhr, 2005). Several purebred swine associations in the United Stated and Canada have developed methods of selection based on multiple traits compiled into an index using Estimated Breeding Values (EBV) which result in Expected Progeny Differences (EPDs) (Robinson and Buhr, 2005). Modern producers as well as genetic companies mate and isolate superior animals who excel in performance, structural conformity, carcass quality, as well as performance indexes in order to provide genetic diversity to swine producers (Knox, 2011). With increasing pressure to

minimize production costs, swine producers have transitioned to artificial insemination due to economic implications, increased reproductive efficiency and reduced labor costs associated with a reduction in boar numbers at the farm level (Flowers and Alhusen, 1992; Gerrits et al., 2005). Approximately six to seven sows can be mated from a single ejaculate, in contrast to a natural mating when only one sow may be mated at a time (Flowers and Alhusen, 1992; Willenburg et al., 2003a). Boars in a regular collection rotation may be able to produce 40 doses per week (20 sows/week), equaling approximately 2,000 sows per year where boars used for natural service may only be used to mate approximately 6 times per week (Knox et al., 2008; Knox, 2011). This increased mating efficiency as well as advances in processing, packaging and extender technology allow for the wide spread use of superior genetics across the swine industry (Gerrits et al., 2005).

An added benefit to AI is a biosecurity measure in order to minimize disease transmission by limiting the introduction of new males to the breeding herd. The ability to control reproductive diseases is one of the driving forces for implementing artificial insemination in the swine industry (Gúerin and Pozzi, 2005). However, artificial insemination, including that with frozen-thawed boar sperm, does not completely eliminate disease transmission, as there are numerous bacterial and viral diseases that are transmitted through artificial insemination and as easily as semen may disseminate superior genetics, contaminated semen may just as easily spread pathogens (Guérin and Pozzi, 2005). Contaminated boar sperm often contains pathogens responsible for drastic reproductive failures (i.e. infertility in boars and sows, and abortions), which cause considerable economic loss (Guérin and Pozzi, 2005). Several severe bacterial pathogens are transmitted through artificial insemination, including brucellosis, chlamydiosis, and leptospirosis that result in abortion and stillborns in females, and infertility in males (Maes

et al., 2008). In contrast to bacteria, viral pathogens can be spread primarily by semen which cause infertility among males as well as larger systemic implication on herd health. Viruses including the porcine reproductive and respiratory syndrome (PRRS) virus, porcine circovirus II (PCV2), and porcine parvovirus (PPV) do not strictly affect the reproductive cycle of pigs, but also result in reduced growth and performance and a higher mortality rates responsible for massive economic loss for the swine industry (Althouse and Rossow, 2011). Policies such as the inclusion of antimicrobial agents in semen extender to minimize bacterial transmission, the introduction of only negative titer animals in the breeding herd, mandatory quarantine period prior to introduction, and vet-to-vet communications should be employed to ensure herd health (Althouse and Lu, 2005; Althouse and Rossow, 2011; Guérin and Pozzi, 2005).

In recent years, considerable research has evaluated the potential for improvement of the global swine population through genetic selection as well as transgenic technologies. Special attention has been given to identify animals resistant to pathogens such as *E. coli* as well as porcine reproductive and respiratory virus (PRRS), pseudorabies, and porcine circovirus II (PCV2) (Gerrits et al., 2005). With the appearance of genetic disorders including PSS (porcine stress syndrome) and RN (halothane gene), the industry has examined genetic selection techniques in an attempt to limit the propagation of these genes in the future (Robinson and Buhr, 2005; Safranski, 2008). Recent research has focused on improving or altering physiological traits such as carcass composition, focusing on alleles mediating insulin like growth factor (IGF-1) expression, increasing milk production through the expression of α-lactalbumin, and increasing feed efficiency through the expression of phytase secretion in saliva to allow for digestion of phosphorus (Gerrits et al., 2005; Marshall et al., 2006).

3.2 Physiological responses to artificial insemination

3.2.1. Sperm transport following insemination

The ultimate purpose of insemination is to ensure a sufficient number of sperm reach the uterotubal junction (UTJ) and establish a sperm reservoir prior to ovulation in order to fertilize oocytes (Roca et al., 2006). Prior to fertilization, the spermatozoa are exposed to several environments between the cervix and the oviduct of the female reproductive tract (Rodríguez-Martínez, 2007; Rodríguez-Martínez et al., 2005). A large population of sperm must be deposited using transcervical insemination due to the high number of spermatozoa lost from semen backflow from the cervical canal. Approximately 30-40% of inseminated spermatozoa and 70% of inseminated volume will flow back out of the female reproductive tract within an hour following insemination (Mezalira et al., 2005; Rodríguez-Martínez, 2007; Rodríguez-Martínez et al., 2005; Steverink et al., 1998). Spermatozoa not lost through back flow must navigate the sow's cervical folds and uterus in order to reach the UTJ and establish a functional sperm reservoir. Following insemination, spermatozoa that escape phagocytosis and uterine backflow undergo rapid transport through the sow uterus. Uterine myometrial contractions assist in the transport of spermatozoa to the uterotubal junction, which are often mediated by reproductive hormone levels (Rodríguez-Martínez et al., 1982).

3.2.2 Immune response following insemination

As with any addition of foreign materials into the body, inseminations both natural and artificial evoke a massive immune response that may compromise the ability of the sperm to

navigate the uterus and establish pregnancy (Roca et al., 2006). Though billions of sperm cells are inseminated, immune responses and semen backflow eliminate approximately 90% of inseminated sperm from the female reproductive tract, limiting the sperm available to form a functional sperm reservoir and drive fertilization (Roca et al., 2006; Steverink et al., 1998; Vazquez et al., 2005). Upon insemination, the sow's immune system is primed, recognizes spermatozoa to be "non-self," and releases leukocytes into the lumen of the uterus (Matthijs et al., 2003). This response begins approximately 30 minutes following insemination and escalates over the next 2-3 hours (Pursel et al., 1978). Though it has been reported that inseminations occurring within 24 hours of ovulation using liquid semen are optimal in a single insemination system, when utilizing two inseminations, it is hypothesized that the first insemination evokes an acute inflammatory response that may last up to 20 hours (Kaeoket et al., 2003b; Schuberth et al., 2008).

The immediate contact of semen with the female reproductive tract, in particular the uterus, stimulates a series of immunological responses that mediate several reproductive events such as the ovulation process, spermatozoa selection, endometrial tissue priming, and early immunological support for embryonic development (Schuberth et al., 2008). In addition to the spermatozoa, components of seminal plasma and semen extenders are known to escalate the response due to the heterogeneous components, as well as cytokines, eicosanoids, and prostaglandins and components in semen extenders stimulating significant neutrophil migration to the uterus (Lessard et al., 2003; Schuberth et al., 2008). The massive influx of leukocytes ultimately leads to the phagocytosis of sperm cells (Matthijs et al., 2003; Woelders and Matthijs, 2001). Interestingly, spermatozoa that are aged, dead, capacitated, or damaged due to cryopreservation are preferentially targeted for phagocytosis (Pursel et al., 1978). This implies

that the immunological response following insemination aids with sperm selection for transport to the utero-tubal junction for reservoir establishment and fertilization (Schuberth et al., 2008; Woelders and Matthijs, 2001). Phagocytosis accounts for a significant reduction in spermatozoa in as few as 4 hours following mating, with only 1% of the inseminated 3 billion spermatozoa remaining. Recent research has attempted to address factors mediating phagocytosis, indicating that the addition of caffeine and calcium chloride ($CaCl_2$) to artificial insemination doses showed a marked reduction in leukocyte recruitment and phagocytosis 4 hours following insemination (Matthijs et al., 2003). These immunological responses have both long and short term effects on the female reproductive tract and fertilization process (Schuberth et al., 2008), as well as the interaction between polymorphonuclear (PMN) leukocytes and cytokines with the endometrial cells of the uterus (Lessard et al., 2003; Vazquez et al., 2005).

3.2.3 Sperm reservoir establishment

In sows, oocytes are fertilized shortly after ovulation, although spermatozoa are often inseminated well before ovulation. The sow's oviduct provides an environment that allows for the transport, storage and capacitation of sperm, while it also allows the maturation of the oocyte and fertilization (Petrunkina et al., 2001; Rodríguez-Martínez, 2007). In order for sperm to survive and maintain the ability to fertilize oocytes, spermatozoa form a sperm reservoir (SR) in the infundibulum of the oviduct. There, sperm remain in a quiescent state and undergo functional changes including destabilization of the cell plasma membrane, otherwise known as capacitation and hyperactivation (Holt, 2011; Rodríguez-Martínez, 2007; Rodríguez-Martínez et al., 2005; Talevi and Gualtieri, 2010). Both functional changes appear to be required in order to induce the

acrosome reaction in presence of the zona pellucida and to then penetrate the oocyte (Rodríguez-Martínez, 2007; Tienthai et al., 2004). Sperm capacitation does not occur *in vivo* in the pre and peri-ovulatory sperm reservoir, but capacitation increases quickly after ovulation (Tienthai et al., 2004). Evidence has shown that sperm's binding to the oviduct in the pig is extremely selective, exhibiting preferences toward uncapactiated sperm (Fazeli et al., 1999) with normal morphology and absence of cytoplasmic droplets (Petrunkina et al., 2001). Additionally, there is clear evidence supporting differences among individual boars, indicating significant boar-to-boar variation (Petrunkina et al., 2001). Additionally it should be noted that spermatozoa that are old, dead, or weakened from cryopreservation are represented in significantly lower numbers in the oviduct of the sow (Einarsson and Viring, 1973; Pursel et al., 1978).

Though mechanisms regarding sperm release are still unclear, experiments have reported the progressive and continuous release of sperm from the sperm reservoir in the oviducts occurs prior to ovulation. This process increases after ovulation and may be related to the induction of capacitation of spermatozoa due to their exposure to oviductal fluid (Rodríguez-Martínez, 2007; Talevi and Gualtieri, 2010) allowing for a low number of capacitated sperm available in the oviduct at the time of ovulation, as sperm in a capacitated state are short lived and eventually undergo cell death (Rodríguez-Martínez, 2007). A gradual release of spermatozoa would indicate pre-ovulatory endocrine changes within the oviduct, such as increasing progesterone and decreasing estrogen, which would impact the release of spermatozoa from the oviductal crypts (Talevi and Gualtieri, 2010). Pre- and post-ovulatory release of spermatozoa is beneficial for species that show variation in time of ovulation in relation to insemination or natural mating, to ensure sperm capable of fertilization are in the oviduct at the time of ovulation (Rodríguez-Martínez, 2007; Rodríguez-Martínez et al., 2005; Talevi and Gualtieri, 2010). Further

knowledge of the physiological function of sperm reservoir establishment in the oviduct will be useful in developing assays in order to determine spermatozoa with superior fertilizing capacity.

3.3 Artificial insemination with frozen-thawed boar sperm

3.3.1 Applications for frozen-thawed boar sperm

Frozen-thawed boar sperm has been commercially available since 1975 and utilized for the transfer of specific genetics both domestically and worldwide (Gerrits et al., 2005). Other domestic livestock species, such as dairy and beef cattle, have utilized the advantages of frozen-thawed sperm, building the industry around the use of superior genetics to drive genetic change. However, cryopreservation of boar sperm has had challenges due to sensitivity of the sperm cell to temperature and osmolarity changes, yielding lower post thaw quality and in turn fertility (Watson, 2000). The use of frozen thawed boar sperm is currently limited to research, international and niche markets. However, refinement of cryopreservation, semen evaluation, and artificial insemination protocols may allow for practical and efficient use of FTS in the swine industry.

Swine producers internationally with less advanced genetics can benefit from novel genetics. However, there are limitations for the distribution of liquid boar sperm internationally. While there have been advancements in extender technologies, liquid extended semen still has a finite lifespan limited to short, (1-3 days), medium (3-5 days) and long-term (5-10 days) (Gerrits et al., 2005; Levis, 2000; Vyt et al., 2004). Additionally, variation in transport including time of transit, temperature, pressure and handling procedures all influence the semen quality and

fertility, making international distribution without detrimental effects to semen quality difficult (Knox, 2011). The loss of fertility due to fluctuations in shipping conditions has driven the transition to frozen-thawed boar sperm artificial insemination in Norway, where producers prefer to have semen doses on site at time of estrus and not reliant on standard delivery methods (Almlid and Hofmo, 1995; Hofmo and Grevel, 2000). Since the early 1980s, Norway has utilized frozen-thawed boar sperm for artificial insemination, accounting for 90% of all matings. This advantage of frozen-thawed boar sperm may also apply to nations in South America and Asia with limited infrastructure, in which intensive swine production has been rapidly increasing as pork is an inexpensive form of dietary protein.

Cryopreservation of boar semen allows for the indefinite storage of genetic material for repopulation, reestablishment of genetic lines following a disease outbreak, expansion of swine herd genetic pools, safeguards against environmental changes, and for research applications (Almlid and Hofmo, 1995; Bailey et al., 2008; Purdy, 2008). Similar strategies are currently employed in many agricultural species including beef and dairy cattle, horses, sheep and goats (Gandini et al., 2007). Organizations such as the USDA-ARS National Animal Germplasm Program (NAGP) preserve genetics for agricultural species in the United States (Purdy, 2008). However, private genetic companies and boar studs have also begun banking cryopreserved semen samples, keeping an inventory that dates back decades (Roca et al., 2006). However, the complete breed reconstruction using frozen-thawed boar sperm alone may be rather costly and take many years to reestablish a genetic line (Gandini et al., 2007).

Increased protection against disease transmission is another advantage with frozen-thawed boar sperm, as it is with liquid extended boar sperm. In most cases, producers must weigh the options of the introduction of new boars into the nuclear herd to prevent inbreeding,

while limiting the introduction of new animals and pathogens they may carry (Knox, 2011). Cryopreservation of boar sperm prior to shipment and mating allows for time to screen parental males for latent bacterial and viral infections (Knox, 2011). Some tests yield results in 1-2 days, in the case of viruses such as PRRS, however many bacterial infections may take longer to detect, be more difficult to treat, and be shed in semen after an active infection has cleared leaving the possibility of disease transmission (Guérin and Pozzi, 2005; Maes et al., 2008).

3.3.2 Limitations of frozen-thawed boar sperm use

Though there are some realistic advantages for frozen-thawed boar sperm, there are some serious drawbacks. Johnson et al. (2000) reported that less than 1% of all matings in the United States occur with frozen-thawed boar sperm, primarily due to the variable fertility results when compared to liquid extended semen (Johnson, 1985a) and reduced fertile lifespan once inseminated (Einarsson and Viring, 1973; Waberski et al., 1994a). According to PigCHAMP records for 2010, the average farrowing rate for sows inseminated with liquid extended semen is 82% with an average litter size of 12.8 pigs/litter (PigCHAMP, 2010) while typical FTS farrowing rates are ~70% with 10 pigs/litter (Almlid et al., 1987; Johnson, 1985b; Roca et al., 2006) However, recent AI and cryopreservation procedures have shown evidence of increased fertility. Higher production costs of frozen-thawed boar sperm is also a deterrent for widespread commercial use, with freezing and production costs alone costing double most liquid insemination doses, resulting from increased labor and supplies (Spencer, 2010). Damage to sperm cells associated with the cooling, freezing, and thawing procedures leads to the need for a significant increase in spermatozoa per insemination, while still yielding lower farrowing rates

and litter size. Major factors producers need to consider when utilizing artificial insemination using frozen thawed boar sperm are boar fertility, sow management and timing of artificial insemination.

3.3.3 Historical research of artificial insemination with frozen-boar sperm

Preliminary attempts to obtain pregnancies with frozen-thawed boar sperm prior to 1970 were unsuccessful (Bwanga, 1991; Johnson, 2000). In 1970, Polge showed that surgical insemination of $70x10^6$ motile frozen-thawed sperm directly into the sow oviduct yielded pregnancy, showing cryopreserved boar sperm was capable of fertilizing oocytes and sustaining pregnancy (Polge et al., 1970). Pursel and Johnson (1975) were able to obtain 85% fertilization rate through the use of heterospermic inseminations, known as mixed sire matings, using sperm with >40% motility at time of insemination. Extensive research has been conducted to continually refine the cryopreservation procedure as well as insemination dosage, timing, and methodology (Johnson et al., 2000; Maxwell and Salamon, 1977, 1976). A majority of experimentation evaluating the optimal number of spermatozoa necessary for pregnancy establishment utilizes $5x10^9$ total sperm cells (~ $2x10^9$ motile sperm cells) (Johnson, 1985b). However, research improving post-thaw motility as well as the implementation of intrauterine inseminations has dramatically lowered the minimum number of spermatozoa necessary for fertilization (Eriksson et al., 2002; Vazquez et al., 2008). Original frozen-thawed boar sperm research concentrated on the importance of multiple matings, in most cases using two to four matings per standing estrus. The literature has favored multiple inseminations for farrowing rates, however, there is little effect on litter size (Almlid et al., 1987; Kemp and Soede, 1996;

Martin et al., 2000; Xue et al., 1998). This preference towards a multiple mating system helps account for variation in ovulation and the limited lifespan of frozen-thawed sperm cell (Johnson, 1985b; Waberski et al., 1994a).

Recent research has focused on the importance of the post thaw quality of frozen-thawed boar sperm as well as compounds that improve these quality measures. Casas et al. (2010) indicated that the quality of semen is more influential in frozen-thawed boar sperm than with fresh semen, exhibiting substantial differences in pregnancy rates between good and poor quality freezing boars with frozen semen compared to AI with fresh. Though known to be detrimental during the cryopreservation process, seminal plasma and its advantages have been explored as a supplement for insemination doses, however, results of these experiments have been mixed, showing a slight advantage in conception rates and litter sizes for poor freezing boars (Abad et al., 2007a; Abad et al., 2007b; Okazaki et al., 2009b). Kirkwood et al. (2008) stated that the addition of seminal plasma to frozen thawed ram semen actually prevented or reversed the cryo-capacitation phenomenon observed with frozen-thawed sperm and further increased fertility. Further, additives such as caffeine and calcium chloride ($CaCl_2$) to extended semen has shown a significant reduction in a post-insemination immune response and lower rate of phagocytosis (Matthijs et al., 2003; Woelders and Matthijs, 2001). Hormonal supplementation research has been conducted with the addition of prostaglandins to insemination doses and exogenously to sows and has shown increased farrowing rates and litter sizes when used with liquid extended semen and is believed to have the same potential when used with frozen-thawed boar sperm (De Rensis et al., 2011; Kos and Bilkei, 2004; Willenburg et al., 2003a).

3.3.4 Advanced reproductive techniques employed with frozen-thawed boar sperm

The current standard for artificial insemination in swine is for a large number of spermatozoa (>2-3 x 10^9 cells) to be delivered through the cervix, however, this is extremely inefficient and limits the ability to disseminate superior male genetics into a large number of females (Martinez et al., 2002; Roca et al., 2011; Roca et al., 2006; Vazquez et al., 2005; Vazquez et al., 2008). Due to this drive for reproductive efficiency, the swine industry has searched for alternatives to conventional cervical artificial insemination that will allow for fewer spermatozoa to be inseminated and for the sperm to circumvent the cervix (Martinez et al., 2002; Vazquez et al., 2005). Producers have explored using intrauterine and deep intrauterine insemination capitalizing on the ability to decrease the number of spermatozoa and extender per insemination, thus increasing the efficiency of artificial insemination with both liquid and frozen-thawed semen (Martinez et al., 2002; Vazquez et al., 2005; Vazquez et al., 2008). The adoption of these techniques may help with sex-sorted or cryopreserved boar semen and help increase in vivo fertility.

In an attempt to increase the effectiveness of sperm used for artificial insemination, research has been conducted on the application of intrauterine (IUI) and deep intrauterine (DIUI) artificial insemination (Roca et al., 2011; Roca et al., 2006; Vazquez et al., 2005; Vazquez et al., 2008). Depositing spermatozoa deeper in the female reproductive tract may allow the sperm to overcome the issues reported with conventional intracervical insemination, such as the sperm loss due to issues of transport through the cervix and phagocytosis following insemination (Martinez et al., 2005; Tummaruk and Tienthai, 2010). Additionally, the use of IUI and DIUI allows for the direct delivery of sperm into the uterine body for IUI and a uterine horn

in the case of DIUI. This can allow for the reduction of the number of sperm needed in each insemination, while still ensuring a sufficient number of sperm in the uterine tubal junction prior to ovulation (Roca et al., 2011; Watson and Behan, 2002). With deeper semen deposition, there is the ability to reduce the amount of sperm inseminated with as low as 1 billion with IUI and 150 million with DIUI which increases the amount of females mated from one ejaculate (Martinez et al., 2001; Tummaruk and Tienthai, 2010; Vazquez et al., 2005). In order to demonstrate the magnitude of this increase, while most boars can produce 2,000 artificial insemination doses (1,000 sows) per year with 3 billion cells, the reduction of sperm numbers to 500 million utilizing DIUI would increase the number of doses produced by 600% (Mezalira et al., 2005). As in the case with trans-cervical AI, much of the success of IUI and DIUI is still vastly dependent on proper insemination dosage and timing relative to ovulation. Bolarín et al. (2006) demonstrated the importance of insemination dosage and timing in relation to ovulation using DIUI where it was observed that sows inseminated with 2×10^9 sperm cells showed a 20% increase in farrowing rate when mated pre- and peri-ovulation compared to post-ovulation, indicating the importance of insemination prior to ovulation. Watson and Behan (2002) examined the practicality of IUI in a large commercial sow operation (3240 sows) that compared cervical insemination and IUI while using 1, 2 and 3 billion sperm cells per insemination. It was noted that comparable pregnancy rates were observed for all treatments (88.7-92.6%), with the exception of 1×10^9 sperm cells using a cervical insemination (66.2%). This experiment illustrates the ability to reduce spermatozoa numbers by one-half to two-thirds using IUI. The main shortcoming of IUI and DIUI leading to its limited usage in the commercial swine production is the potential damage to the reproductive tract (Martinez et al., 2002, 2001; Watson and Behan, 2002) attributed to the type of rod and operator experience (Vazquez et al., 2008).

The introduction of IUI and DIUI has allowed for experimentation with sex sorted sperm, sperm cryopreservation, and the development of sperm mediated gene transfer for the production of transgenic animals have changed the focus of research in the swine industry. In most cases, these advanced processing procedures damage sperm cells, compromising their fertilizing ability and fertile lifespan. In particular, development of gender selection of spermatozoa has been a recent trend in the swine industry in the advancement of breeding programs and reproductive efficiency allowing for gender pre-selection for genetic companies and seedstock operations, as well as animal welfare regulations on castration and animal handling (Bailey et al., 2008; García et al., 2007). Sex-sorted spermatozoa technology has been heavily utilized in the beef and dairy cattle industry through the successful integration of cryopreservation and flow cytometry, however the flow-cytometer does cause a considerable amount of damage which is compounded with damage from the cryopreservation process (Bailey et al., 2008; García et al., 2007). In swine, litters produced using sex-sorted sperm have been born through in vitro fertilization, embryo transfer, and direct deposition into the oviduct (Bailey et al., 2008; Bathgate et al., 2007).

Perhaps the most novel advancement to be utilized with frozen-thawed boar sperm in recent years is the introduction of ovulation induction drugs. Timing of insemination relative to ovulation is one of the most crucial factors to ensure fertilization and pregnancy establishment, especially when utilizing frozen-thawed boar sperm (Einarsson and Viring, 1973; Waberski et al., 1994a). Early research involving ovulation induction drugs centered around the utilization of porcine LH and hCG with the expected time of ovulation occurring between 38 and 42 h after the onset of estrus (Abad et al., 2007a; Bertani et al., 1997; Hunter and Dziuk, 1968). However, recently Stewart et al. (2010) and Knox et al. (2011) have shown that intravaginal administration

of triptorelin given after weaning will advance and synchronize ovulation. The ability to better predict time of ovulation may help compensate for the shorter *in vivo* lifespan of frozen-thawed boar sperm yielding higher pregnancy rates and litter size. Additionally, the ability to induce ovulation combined with intrauterine insemination methodology may also make a single insemination system possible without sacrificing fertility, thereby increasing the efficiency of artificial insemination.

3.4 Optimal timing of artificial insemination

The success of artificial insemination in swine is essentially dependent on optimal timing. While many sources of variation can limit the efficiency of AI, the interval from onset of estrus to the time of ovulation is the most critical component in maximizing fertility (Weitze, 1995; Weitze et al., 1994). For this reason, extensive research has been conducted on the factors affecting the variation in time of ovulation in relation to onset of estrus showing that ovulation occurs approximately 75% through estrus in sows at approximately 32-48 hours (Kaeoket et al., 2002; Steverink et al., 1997), with ovulation accelerated in gilts, occurring at 30-40 hours after the onset of estrus (Almeida et al., 2000; Bracken et al., 2003; Nissen et al., 1997; Soede et al., 1995; Weitze et al., 1994). This knowledge should be utilized to adapt alternative mating strategies for gilts and sows to accommodate the variation in ovulation (Steverink et al., 1999).

It is well accepted that sows mated at intervals greater than 24 hours prior to ovulation have been shown to have lower farrowing rates and litter sizes than those inseminated closer to ovulation (Almeida et al., 2000; Nissen et al., 1997; Soede et al., 1995). However, research has also shown that gilts are restricted to a 12 hour period prior to ovulation to yield optimal

fertilization rates with FTS (Waberski et al., 1994a). Reduced conception rates, lower incidence of normal fertilization and increased embryonic loss are observed when insemination occurs after ovulation (Almeida et al., 2000; Bortolozzo et al., 2005; Hunter and Dziuk, 1968). Oocytes have approximately 8-10 hour lifespan in the oviduct, however, those fertilized late have been shown less likely to develop into viable embryos (Almeida et al., 2000). Approximately 40% of embryonic loss occurs between Day 21-25 of gestation when fertilization occurs 8 hours after ovulation, due to changes in the oviductal environment following ovulation (Almeida et al., 2000). In regards to frozen-thawed boar sperm, it is widely accepted that cryopreserved sperm has a considerably shorter lifespan in comparison to liquid extended semen due to damage associated with the cooling, freezing, and thawing procedures (Bailey et al., 2008; Einarsson and Viring, 1973). With this, the interval from insemination to ovulation is much more sensitive when using frozen-thawed semen, and optimal fertility is observed when insemination occurs within 6 hours prior and 4 hours following ovulation (Okazaki et al., 2009a; Waberski et al., 1994a). Evaluation of post-ovulatory inseminations on fertility has yielded mixed results and the influence on pregnancy rate and litter size when only one mating occurs following ovulation (Castagna et al., 2003; Rozeboom et al., 1997; Waberski et al., 1994b). Though, when all inseminations occur following ovulation, the percentage of sows with normal embryos (Soede et al., 1995) and farrowing rate and litter size (Nissen et al., 1997) all show a marked decline, attributed to the decreased fertile lifespan of oocyte in the reproductive tract after ovulation.

In sows, traditionally, the most accurate indicator of estrus-to-ovulation interval is the weaning-to-estrus interval (Almeida et al., 2000; Belstra et al., 2004; Kemp and Soede, 1996; Knox et al., 1999; Tarocco and Kirkwood, 2001). The wean-to-estrus phenomenon is signified by sows expressing estrus earlier but which ovulate later, while sows that express estrus later

following weaning tend to ovulate earlier (Kemp and Soede, 1996; Knox et al., 1999; Knox et al., 2002; Tarocco and Kirkwood, 2001). It has been demonstrated that regardless of weaning-to-estrus interval, proper timing of artificial insemination in relation to ovulation will allow for fertilization and pregnancy establishment. In addition to weaning-to-estrus interval, other factors have been evaluated for their relationship and influence on estrus-to-ovulation interval. Mixed data has been reported on the influence of seasonal effects (Knox and Rodriguez-Zas, 2001; Peltoniemi et al., 1999; Steverink et al., 1999). Other factors influencing estrus-to-ovulation interval in sows include the interpretation of environmental cues driven by differences in parity (Steverink et al., 1997), genetic lines and breeds (Belstra et al., 2004), and lactation length (Belstra et al., 2004). Steverink et al. (1999) has reported that as much as 23% of variation in estrus duration and estrus to ovulation interval is attributed to farm-to-farm variation due to skill level of employees, sow rearing and nutrition, and boar power.

Though time of insemination relative to ovulation is vital, proper sperm dosage in relation to insemination to ovulation interval is also essential for pregnancy establishment. Regardless of time of ovulation, sufficient sperm concentration at time of AI is crucial to ensure fertilization and sufficient blastocyst formation and ultimately litter sizes (Bracken et al., 2003). Doses consisting of less than 1 billion sperm cells consistently show lower conception rates, blastocyst formation and litter sizes, regardless of proximity of insemination to ovulation, unless the spermatozoa are deposited into the uterine body (Bracken et al., 2003; Spencer et al., 2010). Studies have evaluated the effect of single low dose inseminations at several intervals prior to insemination, and it is well established that sows treated with fewer sperm at 24 h prior to ovulation have significantly reduced fertility (Garcia et al., 2007). With that said, optimal insemination timing can only be achieved through consistent and accurate estrus detection.

Chapter 4. Experiment 1: The effect of temperature and duration of storage of frozen-thawed boar sperm on measures of post-thaw fertility

4.1 Abstract.

Frozen-thawed boar sperm (FTS) is known to have reduced fertility, due in part to a shorter *in-vitro* and *in-vivo* fertile lifespan when compared to liquid extended semen. It is not clear whether alternative storage temperatures following thawing can help to extend the lifespan of FTS. The objective of this experiment was to evaluate *in-vitro* measures of fertility when storing FTS at 17ºC, 26ºC, or 37ºC. Ejaculates (n = 20) from 17 boars were frozen in 0.5 mL straws. All samples were assigned in a 3 X 4 factorial treatment design with samples stored at 17, 26, and 37º C for 2, 6, 12 and 24 h. One straw from each ejaculate was thawed at 50ºC for 20 s and diluted into 10 mL of Androhep Thaw Extender® at 26ºC, to a final concentration of 7 x 10^8 sperm/mL. A sub-sample was evaluated at 0 h (30 min) at 37 °C to establish pre-treatment fertility measures. The extended sample was then divided into 3 mL aliquots and placed at 17ºC, 26ºC, or 37ºC. Each tube was sub-sampled during storage. Motility was evaluated by counting 10 fields at 200 X and viability determined using propidium iodide. Analysis was performed using MIXED procedures for a factorial design for the effects of temperature, duration of storage, and boar. Pre-treatment fertility measures averaged 48% motile and 52% viable sperm. There was an interaction of temperature and duration of storage for motility and viability ($P <$ 0.001). Motility and viability remained >40% during the 2 h storage period. At 6 h, samples stored at 37ºC, showed a 35% decline in motility and viability, while samples stored at 17ºC and 26ºC declined by 10%. Storage of FTS for 12 h resulted in additional loss of 20% for 37ºC samples, and a 5% loss for samples held at 17ºC or 26ºC samples. By 24 h, all samples stored at

37°C were non-motile with < 5% viability, while samples stored at 17°C or 26°C averaged 20% motility and viability. Interestingly, there was an individual boar response to treatment (P < 0.001). These results indicate that FTS can be extended and held at 17°C or 26°C for up to 2 h before use; allowing for preparation and storage of multiple doses at one time. Storage of FTS at 37°C is detrimental to *in-vitro* fertility, but may be useful help to predict *in-vivo* sperm survival.

4.2 Introduction

In modern swine production, there is limited use of frozen-thawed boar sperm (FTS) for artificial insemination due to the variable pregnancy rates and litter sizes resulting from FTS artificial insemination (Johnson, 1985b; Johnson et al., 2000). The shortened lifespan of FTS in comparison to liquid extended semen has been well documented both *in vivo* and *in vitro* and is assumed to be the cause of lower fertility when used in artificial insemination (Einarsson and Viring, 1973; Pursel et al., 1978; Roca et al., 2006; Waberski et al., 1994a). It has been hypothesized that many aspects of post-thaw handing procedures have large implications on quality measures such as motility, viability and acrosome integrity. There has been extensive research on the importance of pre-freeze semen handling and temperature to determine the optimal pre-freeze storage temperature and duration (Guthrie and Welch, 2005; Johnson, 1985b; Watson, 1995), cooling rate (Pursel et al., 1973; Pursel and Park, 1985), freezing rate (Almlid et al., 1987; Hernández et al., 2007c), and thawing rate (Fiser et al., 1993; Thurston et al., 2001), to ensure high quality post-thaw. However, there is little available information on the importance of post-thaw semen handling and its implications on *in vitro* and *in vivo* fertility. The objective of this experiment was to evaluate *in vitro* measures of fertility, motility and viability, when

storing FTS at 17 °C, 26 °C, or 37 °C for up to 24 h. In this experiment, the storage temperatures were selected due to their practical implications in the swine industry, with 17ºC being the storage temperature for liquid semen, 26ºC as approximate room temperature, and 37ºC for the temperature at which semen evaluation is conducted and also as a representation of the approximate physiological temperature of the pig.

4.3 Materials and methods.

4.3.1 Semen collection, thawing, and assignment.

Ejaculates (n = 20) from 17 boars of different breeds, that were in a continuous collection and freezing rotation, were collected from February 2010-June 2011. Ejaculates were extended 1:1 in Modena Extender (Swine Genetics International, Cambridge IA) and shipped overnight and frozen in 0.5 mL straws at the University of Illinois. Samples were stored in liquid nitrogen until evaluation. For detailed procedures of the cryopreservation process, see Appendix A. Straws were thawed at 50°C for 20 seconds. Contents of the straws were diluted into 10 mL of Androhep CryoGuard Thaw Extender® (Minitube of America, Verona, WI). All samples were assigned to a 3 x 4 factorial treatment design with samples stored at 17 °C, 26 °C, and 37 °C in a water bath for 2, 6, 12 or 24 h. An aliquot (1 mL) was removed from each sample to evaluate pre-treatment motility and viability. Extended samples were then divided into 3 mL aliquots in glass culture tubes and stored in digitally controlled thermal devices at 17°C or 26°C or a water bath at 37°C. Each treatment tube was sub-sampled (500 μL) at 2, 6, 12, and 24 h during storage.

4.3.2 Semen evaluation.

Post-thaw evaluation was conducted manually using procedures for motility and viability described in Appendix B. Samples were evaluated at specified times during storage. A minimum of 10 fields and 100 cells were observed at 200X for motility. Fluorescent staining with propidium iodide (Sigma Aldrich, St. Louis, MO) was conducted on all samples in order to establish membrane integrity. Samples were diluted 1:50 in Beltsville Thaw Extender (Minitube of America, Verona, WI) and 7 μL propidium iodide were added. Samples were incubated with the fluorescent probe for 10-15 minutes at 26°C. Samples were fixed with 0.4% paraformaldahyde in PBS and observed using a captured image using a propidium iodide filter of the Carl Zeiss AxioCam HRc (Carl Zeiss Microscopy, LLC. Thornwood, NY). Samples were evaluated using a bright field image and a propidium iodide image of 200 total cells and percentage of membrane intact cells was calculated.

4.3.3 Statistical analysis.

Motility and viability data were analyzed using the MIXED procedures of SAS for repeated measures of SAS for a factorial design using the main effects of temperature, duration of storage, and boar.

4.4 Results.

Motility and viability were affected by an interaction of storage temperature and duration of storage, as well as boar ($P < 0.0001$). Pretreatment quality measures averaged 48.6 ± 2.5% for motility and 52.5 ± 2.6 % for viability. There was a storage temperature x duration interaction ($P < 0.0001$) for motility and viability, but the effects were minimal. Storage temperature affected motility with 17 °C and 26 °C storage greater (38.5%) and lowest in the 37 °C (26%) while duration reduced overall motility from 49% at 0 h to 11% at 24 h (Table 4.1). Within the 17 °C and 26 °C treatments, motility remained >40% for 6 h following thawing, but declined between 12 h and 24 h. Samples held at 37 °C exhibited a reduction in motility within 2 h post thaw and were non-motile after 24 h of storage. Viability was greatest in the 17°C and 26°C (41.4%) compared to 37°C (29%) and was affected by duration of storage and decreased from 53% at 0 h to 15% at 24 h (Table 4.2). Within treatments, viability of samples held at 17°C and 26°C was >40% for up to 12 hours of storage, while samples held at 37°C exhibited a decrease at 6 h of storage and gradually declined at 24 h.

4.5 Discussion.

The objective of this experiment was to evaluate *in vitro* motility and viability, when storing FTS at 17 ºC, 26 ºC, or 37 ºC for up to 24 h following thawing. The results indicate that FTS can be extended and held at 17ºC or 26°C for up to 2 h following thawing with no significant decrease in motility and membrane integrity. Motility was >40% following thawing for all storage temperatures, but those samples that were extended and stored at the highest

treatment temperature (37°C) showed a significant and gradual decrease in post-thaw quality at all observed time points. Additionally, it should be noted that viability was considerably less sensitive to storage temperature and duration, with samples held at 17 °C and 26 °C showing a marked reduction at 6-12 h whereas a decline in motility was observed at 2 h. Storage of frozen-thawed boar sperm at 37 °C has detrimental effects on *in vitro* motility and viability within 2 h. A reduction was observed at every time point; leading to samples being non-motile and non-viable by 24 h following thawing.

These results have implications for FTS handling and processing procedures as well as *in vitro* and *in vivo* fertility. Defined parameters for *in vitro* lifespan can help establish guidelines for multiple dose preparation and storage prior to insemination. This can also be used as a more accurate indication of post-thaw quality due to an increased lifespan for evaluation. Additionally, while post-thaw quality results of samples held at 37°C declined quickly, this may be used a sensitive and rapid indicator of *in vivo* post-thaw sperm survival patterns. It is hypothesized that frozen-thawed boar sperm has a considerably shorter lifespan *in vivo* than liquid extended semen (Einarsson and Viring, 1973; Pursel et al., 1978; Roca et al., 2006; Waberski et al., 1994a). Results would indicate that the temperature alone is sufficient to lower post-thaw quality. This experiment, however, does not take into account other *in vivo* necessary aspects of fertilization such as uterine conditions, capacitation, and acrosome reaction status.

4.6 Tables.

Table 4.1
Least squares means for the motility of frozen thawed boar sperm in response to the effect of holding temperature and duration of storage assessed at specified time intervals

Treatment[e]	n[f]	Pre-Treatment motility	Duration of Storage (h)			
			2	6	12	24
17 °C	20	48.6 ± 2.6	49.7 ± 2.6^{ax}	40.8 ± 2.6^{bx}	34.7 ± 2.6^{bx}	19.3 ± 2.6^{cx}
26 °C			50.1 ± 2.6^{ax}	42.4 ± 2.6^{bx}	37.6 ± 2.6^{bx}	13.4 ± 2.6^{cy}
37 °C			43.2± 2.6^{ay}	23.4 ± 2.6^{by}	12.1 ± 2.6^{cy}	1.36 ± 2.6^{dz}
P Value			$P < 0.05$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

(abcd) Within a row, means without a common superscript are different ($P < 0.05$).
(xyz) Within a column, means without a common superscript are different ($P < 0.05$).
[e] There was an interaction between storage temperature and duration of storage on post-thaw motility ($P < 0.0001$).
[f] Number of straws. One straw from each ejaculate from boars (n = 17) was thawed and aliquoted into 3 mL sub-samples.

Table 4.2
Least squares means for the viability of frozen thawed boar sperm in response to the effect of holding temperature and duration of storage assessed at specified time intervals

Treatment[e]	n[f]	Pre-Treatment viability	Duration of Storage (h)			
			2	6	12	24
17 °C	20	52.5 ± 2.5	49.4 ± 2.5^{ax}	46.8 ± 2.5^{ax}	39.1 ± 2.5^{bx}	21.1 ± 2.5^{cx}
26 °C			48.5 ± 2.5^{abx}	43.5 ± 2.5^{bcx}	41.5 ± 2.5^{cx}	18.9 ± 2.5^{dx}
37 °C			40.0 ± 2.5^{by}	28.8 ± 2.5^{cy}	19.0 ± 2.6^{dy}	3.9 ± 2.6^{ey}
P Value			$P < 0.05$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

$^{(abcd)}$ Within a row, means without a common superscript are different ($P < 0.05$).
$^{(xyz)}$ Within a column, means without a common superscript are different ($P < 0.05$).
[e] There was an interaction between storage temperature and duration of storage on post-thaw viability ($P < 0.0001$).
[f] Number of straws. One straw from each ejaculate from boars (n = 17) was thawed and aliquoted into 3 mL sub-samples.

Chapter 5. Experiment 2: Effect of interval between artificial inseminations when using frozen-thawed boar sperm on fertility and fetal paternity in mature gilts

5.1. Abstract

Multiple inseminations are used in swine with AI to compensate for variation in time of ovulation and to ensure that sperm are present close to ovulation. The timing of AI is even more critical with use of frozen-thawed boar sperm (FTS) which has a shorter lifespan. However, there is limited information on the impact of the interval between inseminations on fertility. The objective of this experiment was to evaluate the effect of interval between inseminations and estrus to ovulation interval (EOI) on fertility and the impact of each AI using fetal paternity in mature gilts when using FTS. Semen from 11 boars was collected and frozen in 0.5 mL straws at 1.4 x 10^9 cells/mL. Gilts (n = 191) were synchronized using Matrix® and assigned at estrus to AI treatment: 1) AI at a 4 h interval (34 and 38 h); 2) AI at an 8 h interval (30 and 38 h); or 3) AI at a 16 h interval (22 and 38 h). Ultrasonography was performed to determine time of ovulation. In each treatment, the first and second AI were from unique sires to allow for paternity identification using microsatellite markers. Each sire was represented across first and second inseminations. Gilts received an AI with 2 x 10^9 motile FTS in 80 mL of extender. Pregnancy rate and litter size were assessed at slaughter on d 33 following AI. Interval between inseminations affected pregnancy rate ($P < 0.01$) with AI intervals of 8 (80%) and 16 h (75%) greater than the 4 h interval (55%). The estrus to ovulation interval (EOI) also affected pregnancy rate ($P < 0.001$) with increased pregnancy rate for gilts ovulating by 36 h (85%) compared to those ovulating by 24 (55%) and 60 h (47%) but did not differ from those ovulating at 48 h (74%). Total fetuses (10.7), number of normal fetuses (10.2) and embryo survival (70.4%) were not affected by interval between inseminations or EOI ($P > 0.05$). Interval between

inseminations had no effect on the proportion of fetuses sired from the second AI but there was an interaction of treatment with EOI ($P < 0.05$). Gilts inseminated at 8 or 16 h intervals and with 36 or 48 h EOI showed changes ($P < 0.05$) in the proportion of offspring sired by the second AI while gilts inseminated at a 4 h interval did not. The results of this study indicate that time of ovulation and AI spacing interval impacts the offspring sired by insemination but does not impact the in-vivo fertility of either AI. However, with twice daily estrous detection and double AI with use of FTS, the greatest pregnancy rates occur when inseminations are spaced at 8 or 16 h intervals to compensate for variation in ovulation.

Keywords: Artificial Insemination, Boar, Frozen sperm,

5.2.Introduction

Artificial Insemination (AI) is widely used in commercial swine production with equivalent reproductive success and greater efficiency when compared to natural service. AI allows for the selection and use of males with superior traits in performance and production efficiency to mate a large number of females (Gerrits et al., 2005; Knox, 2011; Robinson and Buhr, 2005; Roca et al., 2006). As of the year 2000, 90% of inseminations in the United States were conducted using liquid, extended semen (Johnson et al., 2000; Weitze, 2000). Though the utilization of liquid stored boar semen has exponentially increased in the United States, the same growth has not been observed for frozen-thawed boar sperm (FTS). While there have been numerous advances in the field of boar sperm cryopreservation and artificial insemination, FTS matings account for less than 1% of all inseminations (Johnson et al., 2000) and its use is limited for niche markets, international distribution, and research applications (Knox, 2011; Roca et al., 2006).

While there are advantages for using FTS, these are often outweighed by limitations such as variable pregnancy rates and litter sizes (Johnson, 1985b; Johnson et al., 2000; Roca et al., 2006). This problem has been attributed to the shortened lifespan of FTS semen *in vivo*. Research has demonstrated that FTS has a shorter fertile lifespan than that of liquid extended semen after insemination (Einarsson and Viring, 1973; Waberski et al., 1994a). However, when performed within 4 h of ovulation, a single AI with FTS can match the fertility obtained with liquid extended semen (Waberski et al., 1994a). The fertility results from timing inseminations relative to ovulation has established that inseminations occurring closest to the time of ovulation results in a higher incidence of fertilization (Dziuk, 1970; Flowers, 1997; Nissen et al., 1997; Soede and Kemp, 1995).

Due to the shortened lifespan of FTS, the timing of insemination relative to time of ovulation is critical for maximizing pregnancy rate and litter sizes (Waberski et al., 1994a; Weitze, 1995). However, this result is often difficult to achieve due to the high degree of variation in the interval from estrus to ovulation (Almeida et al., 2000; Belstra et al., 2004; Knox et al., 1999; Steverink et al., 1999; Steverink et al., 1997; Weitze, 1995; Weitze et al., 1994). Variation in estrus to ovulation intervals among sows has lead to widespread use of a multiple insemination system to ensure sperm are present at time of ovulation. Previous research indicates that ovulation occurs at 32-48 h in sows (Kaeoket et al., 2002; Steverink et al., 1997) and at 30-40 h in gilts (Almeida et al., 2000; Bracken et al., 2003; Nissen et al., 1997; Soede and Kemp, 1995).

One uncertainty in a multiple AI system is the effect of the spacing interval between inseminations on overall pregnancy establishment and litter size as well as the fertility effect of either insemination. This becomes an important issue when considering that an immune response occurs following each insemination resulting in a massive influx of leukocytes into the

lumen of the uterus. The immune response occurs in as little as 30 min from AI and escalates over the next 2-3 h (Matthijs et al., 2003; Pursel et al., 1978; Willenburg et al., 2003b) with leukocytes and neutrophils present for 8-20 hours following mating (Kaeoket et al., 2003a; Schuberth et al., 2008). This response is associated with phagocytosis of aged, dead, damaged, and capacitated sperm (Einarsson and Viring, 1973; Pursel et al., 1978) and may be even more of a concern since use of FTS increases the proportions of these types of sperm (Bailey et al., 2008; Einarsson and Viring, 1973) and the short fertile lifespan of FTS (Einarsson and Viring, 1973; Waberski et al., 1994a) necessitates close intervals between inseminations (Pursel et al., 1978; Waberski et al., 1994a).

The objectives of this experiment were to test the effect of inseminating FTS at various intervals from onset of estrus in relation to time of ovulation on pregnancy rate and litter size. Further, the impact of spacing the inseminations was used to determine the effect on the proportion of fetuses sired by each insemination relative to the time of ovulation.

5.3. Materials and methods

5.3.1 Animal selection and estrus synchronization

The experiment was performed at the University of Illinois swine research farm in replicates from November 2010 to August 2011. All procedures used in this experiment were approved by the University of Illinois Institutional Animal Care and Use Committee. Terminal line gilts (n = 240; Genetiporc USA, Alexandria, MN) were moved from a finishing barn into pens in a gestation building between 157 to 199 days of age. Gilts were observed for estrous expression using the back-pressure test while providing fence-line exposure to a mature boar. After 28 days, gilts that had exhibited estrus (n = 218) were moved into gestation stalls and synchronized by feeding 15 mg/head/day of MATRIX (Altrenogest, Merck, Summit, NJ) as a

top dress for 14 days. Following last MATRIX feeding, gilts were checked once daily for estrus at 1700 h, and then on the fourth day, estrus detection was performed twice daily (0500 and 1700 h).

Upon detection of estrus, gilts (n = 191) were randomly assigned to receive inseminations at 4 h (34 and 38 h), 8 h (30 and 38 h), or 16 h intervals (22 and 38 h). Insemination timing was performed at intervals based on a second AI occurring for all treatments at 38 h after onset of estrus. The timing was based on a predicted time of ovulation interval of 36 - 40 h after the onset of estrus (Bracken et al., 2003; Nissen et al., 1997; Spencer et al., 2010). Transrectal real-time ultrasound (Aloka 500V Tokyo, Japan) was performed at 12 h intervals following initial detection of standing estrus and continued every 12 h until ovulation was confirmed to determine the estrus to ovulation interval (EOI) (Spencer et al., 2010). Inseminations were performed using ejaculates from 11 maternal and terminal line boars. Each sire and ejaculate was represented across all treatments and first and second inseminations within each replicate.

5.3.2 Semen collection, freezing, and evaluation

Purebred boars (n = 11) in a commercial collection and freezing rotation were selected for this experiment. Ejaculates (n = 31) were frozen during February 2010 – June 2011 using a modified procedure from Pursel and Park (1985) . Semen was collected at a commercial boar stud, diluted 1:1 with 37° C Modena extender (Swine Genetics International, Cambridge, IA), cooled to 17° C and shipped overnight to the University of Illinois, IL. The samples were evaluated for motility and concentration upon arrival. Sperm were centrifuged for 12 min at 800 x *g* at 4 ºC and the supernanent aspirated. The sperm pellet was resuspended with Androhep CryoGuard Cooling Extender® (Minitube of America, Verona, WI) to a concentration of 2.8 x

10^9 sperm/mL. Sperm were held at 5° C for 2.5 h and diluted to a final concentration of 1.4 x 10^9 sperm/mL with Androhep CryoGuard Freezing Extender® (Minitube of America, Verona, WI). Straws (0.5 mL) were filled using a semi-automatic filling and sealing machine (Minitube of America, Verona, WI). Straws were placed into the Ice Cube® controlled rate freezer (Minitube of America, Verona, WI) at 2° C and processed using the following freezing curve: Step 1: 2° to -4° C in 3 min (2° C/min), Step 2: -4° to -30° C in 0.87 min (29.9° C/min), Step 3: -30° to -25° C in 1 min (5° C/min), and Step 4: -25° to -140° C in 11.5 min (10° C/min). Following the completion of the freezing program, the straws were placed into containers for storage in liquid nitrogen.

Post thaw evaluation of samples was performed on all frozen ejaculates. Random straws were thawed at 50°C for 20 s and the contents of the straws expelled into tubes at 37 ºC. Samples were diluted 1:40 for motility and at 1:400 for concentration evaluation in Androhep CryoGuard Thaw Extender® (Minitube of America, Verona, WI) at 37°C. Evaluation occurred at 20 and 60 min post-thaw using 10 fields and 100 sperm cells for motility using a phase contrast microscope at 200x magnification. The percentage of motile cells within a straw was used to determine the number of straws needed for an AI dose with 2.0 x $10^9 \pm 0.1$ motile sperm cells.

Fluorescent staining was also performed on all random FTS straws from each ejaculate in order to determine viability, using propidium iodide (PI, Sigma Aldrich, St. Louis, MO), and for acrosome integrity using Fluorescein isothiocyanate lectin from *Arachis hypogaea* (FITC-PNA, Sigma Aldrich, St. Louis, MO). Briefly, FTS were thawed and diluted 1:50 in Beltsville Thaw Extender (Minitube of America, Verona, WI) and incubated with the fluorescent stains for 10-15 min and fixed with 0.4% paraformaldahyde in PBS solution. The stained samples were observed

for fluorescence on a Carl Zeiss AxioCam HRc (Carl Zeiss Microscopy, LLC. Thornwood, NY) and sperm counted based on the bright field image, a PI fluorescence image, and a FITC-PNA fluorescence image using 200 total sperm cells at 200x magnification. The total sperm cells with PI and FITC-PNA staining were calculated. Post-thaw sperm quality measures averaged 52.1 ± 1.2% motile (range: 43-67%), 56.6 ± 1.9% viable (range: 40-77%) and 99.7 ± 0.01% live sperm with intact acrosome (range: 97-100%). Each 0.5 mL straw contained approximately 3.4 x 10^8 motile sperm cells.

5.3.3 Sperm thawing and insemination

The appropriate numbers of straws were thawed in a digitally controlled 50°C water bath for 20 s and the contents of the straws expelled into 80 mL of Androhep CryoGuard Thaw Extender® (Minitube of America, Verona, WI) in a 100 mL plastic AI bottle at 26°C. Within 45 min of thawing, gilts were inseminated using a conventional foamed tipped AI catheter (IMV Technologies USA, Maple Grove, MN) with 2.0 ± 0.1 x 10^9 motile sperm cells (4-7 straws per dose).

5.3.4. Pregnancy status and reproductive tract processing

Gilts were sacrificed between day 31 and 36 of gestation and the reproductive tracts collected and processed for pregnancy status, number of normal fetuses, and number of degenerate fetuses. Fetuses were individually weighed and those with abnormal appearances in color and size that were at least 1 SD below those of the normal fetuses, were classified as degenerate. Fetuses were individually identified and the livers removed for DNA genotyping in order to establish parentage. Ovaries were collected and evaluated for ovarian structures

including and the number of corpora lutea and abnormalities such as inactive ovaries and follicular cysts.

5.3.5. Genotyping

To distinguish the impact of interval between inseminations and EOI, parental identification was performed. Parental identification of fetuses was performed using DNA obtained from the semen of the boars, the blood of the gilts, and the liver of the fetuses. Semen and fetal tissue samples were digested using Proteinase-K (Amreseco INC, Solon, OH), sodium dodecyl sulfate (SDS), and dithiothreitol (DTT). DNA was isolated using the ZR-96 Quick-gDNA (Zymo Research, Irvine, CA) according manufacturer instructions. Based on a set of 30 microsatellite markers recommended by The International Society of Animal Genetics (ISAG) and the Food and Agriculture Organization (FAO), a panel of 14 microsatellite markers was chosen (https://www-lgc.toulouse.inra.fr/pig/panel/panel2004.htm). Primers were synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA). Primers were assigned to polymerase chain reactions (PCR) based on fragment size and fluorescent tag combinations. Fragment analysis genotyping and PCR reactions were conducted in a 12.5 μL reaction volume comprised of 2.5 μL of DNA, 10 μL of PCR media composed of 1 μM forward primers tagged with fluorescently-labeled M13 primer (PET®, NED®, VIC®, or 6-FAM® dye, Life Technologies, Carlsbad, CA), as well as one standard 10 μM reverse primer, containing 2 μL of 10x Buffer, 1 μL of 4 mM dNTPs (Invitrogen, Carlsbad, CA), 0.1 μL of HotStarTaq Polymerase (Qiagen, Valencia, CA). PCR was conducted with an initial denaturing temperature of 95 ºC for 5 min followed by 34 cycles of a three step process of 94 ºC for 1 min, 58 ºC for 1.5 min, and 72 ºC for 1.5 min with a final step of 72 ºC for 1 h. Multiplex PCR products were combined and purified using Promega Wizard® SV96 and sequenced as described by Meyers et

al. (2010). Alleles were identified using GeneMarker® software (SoftGenetics, LLC) and checked manually. Parentage was determined using Cervus® based on parental and fetal allele profiles (Field Genetics LTD).

5.3.6 Statistical analysis

Data were analyzed by ANOVA procedures in SAS (SAS Institute Inc., Cary, NC). Binary response measures were analyzed using PROC GENMOD for the effects of treatment (4, 8, and 16 h), and EOI (24, 36, 48, and 60 h) using the chi-square test. Continuous response variables such as number of normal fetuses, total number of fetuses, embryo survival, and the proportion of fetuses resulting from each insemination were evaluated using the PROC MIXED procedures of SAS and significant effects were identified with an F-Test. Differences between least square means were determined using the T-Test for the fixed effects of treatment, estrus to ovulation interval, and their interaction. Other variables such as replicate, boar, and estrus expression after last matrix feeding were tested and removed if non-significant. Data were evaluated for normality using the PROC UNIVARIATE procedures of SAS.

Since the experiment relied on insemination occurring at fixed times relative to the mean ovulation time of 36 h for gilts, data from gilts assigned to treatment were excluded from analysis if the estrus to ovulation intervals were either abnormally short (< 24 h, n = 2) or abnormally long (> 60 h, n = 14). In addition, gilts (n = 4) were also excluded due the diagnosis of ovarian cysts at estrus or at slaughter.

5.4.Results

5.4.1. Pregnancy rate

The pregnancy rates of gilts inseminated at 8 and 16 h were greater than those inseminated at 4 h intervals ($P < 0.05$, Table 5.1). The EOI also affected pregnancy rate ($P < 0.001$, Table 5.2) with gilts that ovulated by 36 h after the onset of estrus having greater pregnancy rates than those that ovulated by 24 and 60 h after onset of estrus but did not differ from those ovulating by 48 h. There was no interaction of interval between insemination and EOI on pregnancy rate ($P > 0.10$).

5.4.2. Number of fetuses

There was no effect of treatment or EOI on total or number of normal fetuses and embryo survival ($P > 0.10$, Tables 5.1 and 5.2).

5.4.3. Proportion of litter and total number of fetuses resulting from first and second inseminations

There was no effect of interval between inseminations on the proportion or total number of fetuses resulting from the second insemination ($P > 0.10$, Table 5.1) but there was an effect of EOI ($P < 0.0001$, Table 5.2). Gilts ovulating by 48 and 60 h had a greater proportion ($P < 0.01$) of fetuses sired by the second AI compared to those ovulating by 36 and 24 h. However, there was a treatment x EOI interaction ($P < 0.05$) for both the proportion and total number of fetuses sired by the second insemination ($P < 0.05$, Table 5.3). For gilts inseminated at a 4 h interval, there was no effect ($P > 0.10$) on the proportion or total number of fetuses sired by the second AI for gilts ovulating at 24, 36, or 48 h. In comparison, for gilts inseminated at 8 and 16 h intervals, the proportions and numbers changed with EOI. At an EOI of 24 or 36 h, the majority of fetuses

were sired by the first AI, while ovulation occurring at 48 h resulted in litters sired predominantly by the second insemination.

5.5.Discussion

The aim of this study was to determine if interval between inseminations had an impact on fertility in relation to estrus to ovulation interval and whether the fertility of the second insemination was compromised with short AI intervals when using FTS. The study was designed to allow for the determination of pregnancy rate, litter size, and the paternity of the fetuses in relation to interval between inseminations, and estrus to ovulation interval. Our results indicate that FTS inseminations occurring at 8 to 16 h intervals were more successful at compensating for variation in time of ovulation and allowing for establishment of pregnancy when ovulation occurred within normal estrus to ovulation intervals in gilts. In contrast, a 4 h insemination interval resulted in a pregnancy rate that was 20% lower than the 8 and 16 h spaced AI intervals. There was no indication of an interaction between insemination interval and estrus to ovulation interval on pregnancy rate or litter size, suggesting that additional factors may be involved in explaining the reduction in fertility observed with the inseminations at 4 h intervals. Collectively, this and other experiments show that FTS can yield pregnancy rates >70% with litter sizes >10 pigs when used in a double AI system with 2×10^9 motile spermatozoa (Johnson, 1985b; Roca et al., 2006).

In this study, treatments were primarily designed to evaluate the effect of interval between inseminations on pregnancy rate and litter size resulting from use of FTS. Intervals between inseminations are especially important with FTS due to the shorter *in vivo* lifespan of FTS. This problem requires more inseminations (Almlid et al., 1987; Bortolozzo et al., 2005;

Martin et al., 2000), inseminations spaced closer to one another (Martin et al., 2000; Waberski et al., 1994a), or more precise timing of insemination relative to ovulation (Einarsson and Viring, 1973; Waberski et al., 1994a). A primary concern with the intervals between inseminations was the potential consequences of an initial immune response following the first insemination (Kaeoket et al., 2003a; Matthijs et al., 2003; Pursel et al., 1978; Woelders and Matthijs, 2001). Inseminations evoke a massive influx of leukocytes into the uterus of the sow in as little as an hour. This response has been shown to last as little as 4 h but as long as 20 h and is responsible for phagocytosis of sperm cells from the uterus (Matthijs et al., 2003; Schuberth et al., 2008; Willenburg et al., 2003b; Woelders and Matthijs, 2001). Any inseminations occurring after the initial immune reaction may be compromised. We chose to evaluate this effect by determining the proportion and number of the offspring resulting from the second insemination. If an immune response altered sperm survival or fertility, it would be evident as a shift in the proportion of the offspring in relation to the intervals tested between the inseminations. Our results indicated that interval between insemination had no effect on the proportion of fetuses resulting from the second AI, but that there was an effect of estrus to ovulation interval and an interaction of the interval between inseminations and the estrus to ovation interval. The proportion of fetuses resulting from the second insemination has been reported with liquid semen AI in weaned sows (Vesseur et al., 1996). In this study, although time of ovulation was not determined, inseminations occurring at the onset of estrus and 24 h later resulted in 45% of the offspring sired by the second AI. Factors such as genotype, parity, wean to estrus interval and sire also contributed to the variation in proportion of offspring sired within a litter. Other studies have also reported on the proportion of offspring sired from inseminations when using pooled semen or with first and second inseminations from different sires as a method to characterize the *in-vivo*

fertility differences between boars (Dziuk, 1970; Flowers, 1997). In the study by Flowers (1997) sire classification was initially based on *in-vitro* motility differences, which resulted in an increase in the proportion of the litter from sires with high motility, regardless of AI sequence. The lack of a sire effect on fertility in our study was our intent as we selected sires based on an established history of fertility and inclusion in the study upon a threshold post-thaw motility and viability.

When examining the effect of estrus to ovulation interval alone, our results showed that for gilts that ovulated by 24 and 36 h, >60% of the offspring were sired by the first AI, while for gilts that ovulated by 48 h, >60% were sired by the second AI. In the 4 h insemination interval, there was no change in the proportion of the litter resulting from the second AI regardless of ovulation time. However, when intervals between inseminations were at 8 or 16 h, the first insemination sired most of the litter at the 24 and 36 h ovulation times, while the second AI sired the majority for those ovulating by 48 h. These results suggest that the insemination that drives fertilization is not always the one that is closest to ovulation. In this case, gilts inseminated at 4 h intervals would have demonstrated a shift in the proportion of fetuses sired by the second insemination with changing time of ovulation. Further, while a shift was observed in the 8 and 16 h AI intervals, the insemination that sired the majority of the litter was not always the insemination closest to ovulation. In fact, there appeared to be an advantage in most cases for the first AI as 62% of the total offspring were sired by the first AI and 38% by the second. This is exemplified in gilts ovulating at 36 h that were inseminated at 8 and 16 h intervals, while the second AI was 2 h from ovulation, over 60% of the piglets were sired by the first AI that was 6 and 14 h prior to ovulation. The assumption would be that the second insemination would drive ovulation, however, the first insemination accounted for siring 72% of the fetuses, indicating an

extended lifespan of frozen-thawed sperm of the first insemination or an immune response compromising the second insemination.

Variation in estrus to ovulation interval is widely reported (Bracken et al., 2003; Nissen et al., 1997; Soede and Kemp, 1995; Steverink et al., 1997; Weitze, 1995; Weitze et al., 1994) and has been shown to be the cause for improper AI timing (Almeida et al., 2000; Bortolozzo et al., 2005; Nissen et al., 1997; Soede et al., 1995). This is true when using both liquid extended semen (Almeida et al., 2000; Bortolozzo et al., 2005; Nissen et al., 1997; Soede et al., 1995; Waberski et al., 1994a) and FTS (Bailey et al., 2008; Einarsson and Viring, 1973; Okazaki et al., 2009a; Waberski et al., 1994a). As a result, multiple inseminations are standard practice in commercial production (Martin et al., 2000; Xue et al., 1998). It is reported that ovulation in gilts occurs between 33-42 h following onset of estrus, (Almeida et al., 2000; Bortolozzo et al., 2005; Spencer et al., 2010). Previous work from our lab observed an average ovulation time of 33 h using a puberty induced and estrous synchronized gilt model (Spencer et al., 2010). Based on these results, and using a more mature gilt model, we predicted a mean ovulation time of 38 h. From this estimate we fixed the time of the second insemination to occur close to the predicted time of ovulation, relative to onset of estrus. The timing of the first mating was altered to create insemination intervals of 4, 8, or 16 h. In this experiment, the estrus to ovulation interval averaged 37 h, when excluding those that ovulated abnormally early or late. Our data showed that from onset of estrus, 24% of gilts had ovulated between 13 to 24 h, 46% between 25 to 36 h, 26% from 37 to 48 h and 6% from 49 to 60 h. With the timing for inseminations chosen for this experiment, the majority of inseminations occurred within the 10 h period before ovulation. Despite this, variation in ovulation time was clearly evident and had an effect on fertility but also responded to the interval between insemination treatment. Optimal fertility was observed for

pregnancy rate at an 8 h interval and when evaluating an index for fertility that included pregnancy rate and normal fetuses, the 8 h interval resulted in a 38% increase in the pigs produced when compared to the 4 h interval, and a 5% increase in pigs produced compared to the 16 h interval when using FTS.

AI timing has practical implications for commercial use of FTS. AI times must fit with the labor availability in most production settings for the technology to be of practical benefit (Didion and Schoenbeck, 1995; Pursel and Johnson, 1975). As a result, AI intervals with FTS within a working day can vary dramatically when a first AI can occur in either the AM or PM and the second AI follows at the next AM or PM. This different AI timing may result in intervals between inseminations being as short as 4 to 8 h or as long as 18 to 24 h. The spacing of the AI intervals can also vary in relation to estrous detection frequency. However, it is clear from this experiment, above all other factor, optimal spacing of inseminations is important for fertility and depends upon the estrus to ovulation interval.

5.6 Conclusion

The results of this study indicate that when using multiple inseminations with FTS, an interval of 8 to 16 h between inseminations will result in higher greater pregnancy rates with acceptable litter sizes when ovulation occurs within a normal range for gilts. Close insemination interval does not reduce the fertilization rate from the second insemination within a litter. However, interval between inseminations does interact with estrus to ovulation interval and the proportion of offspring sired by the first or second AI changes with ovulation time with longer intervals between AI but not in the short interval.

5.7 Tables

Table 5.1 Least square means for pregnancy rate, normal healthy fetuses, and embryo survival data in response to different intervals between inseminations occurring at 4, 8, or 16 h intervals using 2 x 10^9 motile, frozen boar sperm.

Interval between Inseminations[a] (h)	n[b]	Pregnancy Rate[c]	Total Fetuses/ Litter	Number of Normal Fetuses/Litter	Embryonic Survival (%)	Proportion of Litter AI 1 (%)	Proportion of Litter AI 2 (%)
4 h	58	55.4%[x]	9.8 ± 0.7	9.5 ± 0.7	66.0 ± 5.2	54.9%	44.9%
8 h	55	80.0%[y]	10.8 ± 0.6	10.4 ± 0.6	70.2 ± 4.4	57.3%	41.9%
16 h	60	75.0%[y]	11.2 ± 0.6	10.5 ± 0.6	73.9 ± 4.4	64.2%	34.5%

[a] Inseminations for each treatment occurred at the following times: 4 h (34 and 38 h), 8 h (30 and 38 h), or 16 h intervals (22 and 38 h).
[b] Gilts assigned to treatment (n = 20) excluded from analysis due to abnormal short (<12 h) or long (>60 h) estrus to ovulation interval or ovarian abnormalities (n = 2).
[c] Pregnancy rate was established at day 33-35 of gestation.
[xy] Different superscripts within a column are different ($P < 0.05$)

Table 5.2 Least square means for pregnancy rate, normal healthy fetuses, and embryo survival data in response to estrus to ovulation interval with inseminations occurring at 4, 8, or 16 h intervals with 2 x 10^9 motile, frozen boar sperm.

Estrus To Ovulation Interval[a] (h)	Interval from Second AI to Ovulation[b]	n	Pregnancy Rate[c]	Total Fetuses/Litter	Number of Normal Fetuses/Litter	Embryonic Survival (%)	Proportion of Litter AI 1	Proportion of Litter AI 2
24	-14	49	55.1% [x]	10.1 ± 0.8	9.6 ± 0.7	68.0 ± 5.6	81.8% [x]	16.9% [x]
36	-2	61	85.3% [y]	10.6 ± 0.6	10.2 ± 0.5	68.3 ± 4.0	59.9% [y]	39.9% [y]
48	+10	38	73.7% [xy]	11.6 ± 0.8	11.1 ± 0.7	79.9 ± 5.4	33.0% [z]	64.4% [z]
60	+22	15	46.7% [x]	8.7 ± 1.5	8.0 ± 1.4	51.5 ± 10.9	8.3% [z]	91.7% [z]

[a] Estrus detection and trans-rectal ultrasound was conducted at 12 hour intervals (0500 and 1700h).
[b] Interval from ovulation to fixed second insemination at 38 hours.
[c] Pregnancy rate was established at day 33-35 of gestation.
[xyz] Different superscripts within a column indicate differences ($P < 0.05$)

Table 5.3 Least square means for the proportion of a litter and the total number of fetuses sired in response to interval between inseminations (4, 8, and 16 h) and estrus to ovulation interval (24, 36, and 48 h) when using frozen-thawed boar semen. Paternity identification resulting from AI 1 or AI 2 occurred as a result of use of unique sires for each AI when using 2 x 10^9 motile frozen-thawed boar sperm. There was an interaction ($P < 0.05$) between interval between inseminations and estrus to ovulation interval.

	Interval between inseminations								
	4 h			8 h			16 h		
	Estrus to ovulation intervals								
Response	24 h	36 h	48 h	24 h	36 h	48 h	24 h	36 h	48 h
Proportion of litter (%)									
AI 1[d]	66.7	46.7	51.4	91.7[a]	62.1[b]	18.1[c]	93.8[a]	73.6[a]	25.5[b]
AI 2[e]	33.3	53.2	48.3	6.1[x]	37.7[y]	81.9[z]	4.7[x]	25.8[x]	73.0[y]
Total number of fetuses									
AI 1[d]	4.6	3.6	6.5	9.1[a]	7.7[a]	2.4[b]	7.9[a]	7.8[a]	3.2[b]
AI 2 [e]	2.8	4.5	4.4	0.5[x]	3.7[y]	8.0[z]	1.1[x]	3.1[y]	8.7[z]

[d e] AI 2 was fixed for all treatments at 38 h following the onset of estrus and AI 1 varied by treatment interval with AI 1 occurring at 34 h for the 4 h interval, at 30 h for the 8 h interval, and at 22 h for the 16 h interval. .
Different superscripts within an interval between insemination indicate differences in the proportion or total number of fetuses sired from AI 1 ([abc]) or AI 2 ([xyz]) by estrus to ovulation interval ($P < 0.05$).

Chapter 6. References

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Appendix A: Boar Sperm Cryopreservation Protocol

Semen Processing and Freezing:

1) Upon arriving in lab, process semen by evaluating volume, then pour equal volumes into 250mL centrifuge tubes. Place centrifuge tubes in 17^0 C water baths in Coolatron®.

2) Prepare a 2mL subsample in order to evaluate concentration and motility.
   - Concentration: *Evaluate at a 1:100 Dilution*. Load onto Hemocytometer and count 5 diagonal boxes. Calculate Total Number of Sperm cells in Ejaculate (TSE).
     - [1:10] 100 µL of Sperm with 900µL of *Androhep CryoGuard Extender*
     - [1:100] 100 µL of 1:10 dilution and mix with 880 µL of *Androhep CryoGuard Extender* and 20 µL Formaldehyde
     - ***Calculations***: $(1)(Ave\ Hemo\ Count)(5)(10^2)(10^4)$= Concentration

   - Motility: *Evaluate at a 1:40 Dilution*. Allow to warm in warming block for 20 minutes prior to evaluation. Count total number of motile and linearly motile sperm. Evaluate 10 Field and calculate average motility.
     - [1:4] 100 µL Sperm with 300 µL of *Androhep CryoGuard Extender*
     - [1:40] 100 µL of 1:4 dilution and mix with 900 µL of *Androhep CryoGuard Extender*

3) Allow samples to sit in 17^0 C to equilibrate temperature and pH. Weigh centrifuge tubes to determine volume of sample. (Volume is used to determine TSE)***Total Time in 17^0 C: 1-1 ½ Hours***

4) Centrifuge at 16-17^0 C at 800x G (~1700 rpms) for 12.5 minutes in the 250 mL centrifuge tube

5) Aspirate the supernatant out of the centrifuge tubes leaving the sperm pellet, using the sterile hood with the vacuum and flask.

6) Weigh sperm pellet. Based on the calculated final volume, re-suspend the sperm pellet using the calculated volume of cooling extender (*Androhep Cooling Extender*®) and mix with pipette. Cooling extender and pellet weight should account for 50% of the final volume. *The calculated final volume should result in 1.4 x 10^9 Sperm/mL.
   - *Concentration x Volume = TSE*
   - *TSE / (1.4 x 10^9 Sperm/mL) = Freezable Volume*

7) Place samples and freezing extender/cryoprotectant (*Androhep Freezing Extender*®) in 5^0 C water baths in cold room. Allow to cool slowly over 1 ½ hours. After sample reaches 5^0 C, hold for 45 minutes. ***Total Cooling Time (5^0 C): 2 ½ hours.***

8) Add calculated volume of freezing extender and mix gently. * Should equal total calculated freezing volume.

Appendix A (cont..): Semen Packaging

1) Immediately fill and seal labeled 0.5 mL medium straws in cold room. Shake straw magazine to get air bubble to the middle of the straw.

2) Load freezing program. Allow Ice Cube to reach start temperature of 2^0 C. Open lid and place straw racks in controlled freezer. Allow machine to equilibrate at 2^0 C. Start program.

Freezing Rate Table

Time	Temperature	Slope (Degree/Minutes)
0	2^0 C	0
3 mins	-4^0 C	-2^0 C/ min
.87 mins	-30^0 C	$+30^0$ C/min
1 mins	-25^0 C	-5^0 C/min
11 mins	-140^0 C	-10^0 C/min

3) After cycle has finished, stop machine and remove straw racks.

4) Plunge straws into LN2 and sort straws into 8 straws per goblet and 2 goblets per cane.

5) Transfer to liquid nitrogen tank.

Appendix B: Boar Sperm Evaluation Protocol

Motility Evaluation

1. Thaw straw of semen in water bath at 50°C for 20 seconds. Plunge contents of straw into test tube in 37°C heating block.
2. *Evaluate at a 1:40 Dilution.* Allow to warm in warming block for 20 minutes prior to evaluation. Count total number of motile and linearly motile sperm. Evaluate 10 Field and calculate average motility.
    - [1:4] 100 µL Sperm with 300 µL of *Androhep CryoGuard Extender*
    - [1:40] 100 µL of 1:4 dilution and mix with 900 µL of *Androhep CryoGuard Extender*
3. Incubate in thaw extender at 37◦C for of 10-20 minutes.
4. Place sample on pre-warmed slide and place cover over sample.
5. Allow slide to sit on the warmed stage for 1 to 2.5 minutes.
6. Using a phase-contrast microscope with a 20x power objective, count 10 fields of 10 cells each, recording motile sperm.

Fluorescent Staining

1. Thaw straw of semen in water bath at 50°C for 20 seconds. Plunge contents of straw into test tube in 37°C heating block.
2. Prepare staining media in test tubes at room temperature. (26 °C)
    - 500 µL BTS Extender
    - 7 µL Propridium Iodide (PI)
    - 15 µL Fluorescent Lectin with Peanut Allugitin (FITC-PNA)
3. Pipette 10 µL of stock semen sample into staining media. Return stock sample to 7°C warming block until 60 minute evaluation.
4. Allow samples to set in staining media for 10-20 minutes at room temperature (26 °C).
5. Fix samples by adding 2 µL of 0.4% Formaldehyde solution.
6. Pipette 20 µL of the fixed sample onto a microscope slide and cover with cover slip.
7. Allow slide to sit for 10 minutes to allow sperm cells to settle.
8. Using the Zeiss Axioskop®, evaluate the sample by counting 200 total sperm in multiple fields to determine:
    - Percent Viable: Total Non- FITC or Sperm – Bright Filter
    - Percent Live Acrosome Reacted: Non- stained sperm with green acrosome –
    - FITC Filter
    - Percent Dead/Membrane Compromised: stained (red) sperm – PI Filter

9. Compare sperm cells in imaged for PI and FITC stains to Bright Field images to determine Percent Viable, Percent Live with Acrosome Reaction, Percent Dead/Membrane Compromised.
10. Repeat steps 3-9 at 60 minutes post thaw.

Appendix C: Individual Boar and Ejaculate Data

Table C. 1: Least square means of post-thaw evaluation for motility, membrane integrity (MI), and percent live sperm with intact acrosomes (LAI) for the boars (n = 11) used for the breeding trial. Evaluation was conducted at 20 min and 60 min post-thaw.

Boar	Ejaculates (n)	Boar ID	Breed	Motility-20 (%)	Motility-60 (%)	MI-20 (%)	MI-60 (%)	LAI-20 (%)	LAI-60 (%)
A	1	660	Berkshire	48.0 ± 0.0	44.0 ± 0.0	64.0 ± 0.0	60.0 ± 0.0	100.0 ± 0.0	99.3 ± 0.0
B	10	730	Spot	54.8 ± 2.1	53.5 ± 2.2	56.8 ± 4.2	54.8 ± 3.3	99.6 ± 0.2	99.1 ± 0.3
C	2	968	Pietrain	44.0 ± 1.0	46.5 ± 6.5	60.0 ± 1.7	59.5 ± 8.5	100.0 ± 0.0	100.0 ± 0.0
D	2	1349	Duroc	50.0 ± 6.0	55.0 ± 2.0	52.3 ± 2.0	54.6 ± 2.0	100.0 ± 0.0	99.3 ± 0.0
E	1	2423	Yorkshire	48.0 ± 0.0	46.0 ± 0.0	42.0 ± 0.0	43.0 ± 0.0	99.0 ± 0.0	100.0 ± 0.0
F	2	2477	Yorkshire	58.0 ± 3.0	55.0 ± 3.0	57.5 ± 4.5	59.0 ± 3.0	100.0 ± 0.0	99.5 ± 0.5
G	1	2472	Yorkshire	45.0 ± 0.0	43.0 ± 0.0	54.0 ± 0.0	59.0 ± 0.0	99.0 ± 0.0	100.0 ± 0.0
H	1	2488	Yorkshire	50.0 ± 0.0	44.0 ± 0.0	54.2 ± 0.0	51.7 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
I	5	3345	Landrace	51.4 ± 3.0	51.2 ± 4.9	57.4 ± 3.6	57.3 ± 3.9	99.4 ± 0.2	99.5 ± 0.5
J	1	4528	Hampshire	57.0 ± 0.0	53.0 ± 0.0	63.0 ± 0.0	62.5 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
K	4	4529	Hampshire	55.5 ± 3.9	52.8 ± 3.4	55.7 ± 1.5	66.8 ± 1.0	99.3 ± 0.6	99.3 ± 0.6

Appendix C (cont): Individual Ejaculate Data

Table C.2: Least square means of post-thaw evaluation for motility, membrane integrity (MI), and percent live sperm with intact acrosomes (LAI) for the ejaculates (n=31). Evaluation was conducted at 20 and 60 min post-thaw.

Boar	**Ejaculate**	**Concentration (10^9 Cell/mL)**	**Motility-20 (%)**	**Motility-60 (%)**	**MI-20 (%)**	**MI-60 (%)**	**LAI-20 (%)**	**LAI-60 (%)**
A	1	1.44	48.0	44.0	64.0	60.0	100.0	99.3
B	1	1.33	47.0	51.0	60.0	56.0	99.0	98.0
B	2	1.14	50.0	53.0	52.0	55.0	100.0	100.0
B	3	1.51	56.0	62.0	52.6	53.7	100.0	99.1
B	4	1.19	45.0	47.0	40.0	42.0	100.0	100.0
B	5	1.13	54.0	55.0	56.9	46.9	100.0	100.0
B	6	1.25	67.0	57.0	55.0	65.0	100.0	100.0
B	7	1.19	50.0	42.0	76.4	66.6	99.1	98.9
B	8	1.15	42.0	45.0	43.2	40.0	100.0	98.5
B	9	1.25	60.0	61.0	75.6	61.7	100.0	100.0
B	10	1.75	61.0	62.0	51.1	62.0	98.2	97.6
C	1	1.63	45.0	53.0	77.0	68.0	100.0	100.0
C	2	1.16	43.0	53.0	45.0	51.0	100.0	100.0
D	1	1.30	43.0	53.0	45.0	51.0	100.0	100.0
D	2	1.30	56.0	57.0	59.0	42.6	100.0	98.9
E	1	1.66	48.0	46.0	42.0	43.0	99.0	100.0
F	1	1.16	61.0	58.0	53.0	56.0	100.0	99.0
F	2	1.40	55.0	52.0	62.0	62.0	100.0	100.0
G	1	1.40	45.0	43.0	54.0	59.0	99.0	100.0
H	1	1.59	50.0	44.0	54.2	51.7	100.0	100.0

Appendix C (cont):

Table C.2 (cont.)

Boar	**Ejaculate**	**Concentration (10^9 Cell/mL)**	**Motility-20 (%)**	**Motility-60 (%)**	**MI-20 (%)**	**MI-60 (%)**	**LAI-20 (%)**	**LAI-60 (%)**
I	1	1.32	49.0	42.0	68.0	55.0	99.0	98.0
I	2	1.27	47.0	47.0	50.1	49.5	100.0	100.0
I	3	1.74	59.0	58.0	53.7	51.0	100.0	100.0
I	4	1.36	58.0	67.0	55.2	68.7	99.4	100.0
I	5	1.18	44.0	42.0	52.7	54.7	99.3	100.0
J	1	1.13	57.0	53.0	63.0	62.5	10.0	100.0
K	1	1.22	47.0	56.0	53.0	56.0	100.0	97.0
K	2	1.67	60.0	65.0	59.1	57.1	99.2	97.3
K	3	1.55	42.0	48.0	57.1	56.1	100.0	98.0
K	4	1.35	58.0	53.0	53.4	57.7	100.0	100.0
K	5	1.32	52.0	43.0	59.3	54.2	100.0	98.1

Appendix D: Tissue Digest, DNA Isolation, and PCR Protocols

Tissue Digest

1. Aliquot 75 µL of tissue samples.
2. Add 120 µL of DNA Extraction Buffer, 15 µL Proteinase-K, 24 µL sodium dodecyl sulfate (SDS), and 10 µL dithiothreitol (DTT).
3. Incubate at 60 °C for 12 hours.

DNA Isolation

1. Add 75 µ of tissue digest to 300 µL of Genomic Lysis Buffer ™ in 96 well plate. Vortex well.
2. Transfer 300 µL of mixture to Silicon-A™ Plate and Collection plate. Centrifuge at 2,500 x *g* for 5 min.
3. Add 200 µL of Pre-Wash Buffer to each well. Centrifuge at 2,500 x *g* for 5 min.
4. Add 300 µL of g-DNA Wash Buffer to each well. Centrifuge at 2,500 x *g* for 5 min.
5. Transfer Silicon-A™ Plate onto an Elution Pate. Add 50 µL of DNA Elution Buffer to each well. Incubate for 5-10 minutes at room temperature. Centrifuge at 2,500 x *g* for 5 min.
6. Eluted DNA store at – 20 °C until future use.

PCR Protocol

1. Create PCR master mix using the following tables.

4 Marker PCR	**1X**	**100X**
DNA (Per Well)	3	3
10X PCR Buffer	1	100
4mM dNTPs	0.5	50
P1	0.125	12.5
P2	0.25	25
P3	0.125	12.5
P4	0.25	25
P5	0.125	12.5
P6	0.25	25
P7	0.125	12.5
P8	0.25	25
M13	.25	25
Taq	0.07	7
Optima H20	3.68	368
TOTAL	**10**	**1000**

3 Marker PCR	**1X**	**100X**
DNA	3	3
10X PCR Buffer	1	100
4mM dNTPs	0.5	50
P1	0.125	12.5
P2	0.25	25
P3	0.125	12.5
P4	0.25	25
P5	0.125	12.5
P6	0.25	25
M13	.25	25
Taq	0.07	7
Optima H20	4.15	415.5
TOTAL	**10**	**1000**

Appendix D (cont.):PCR Protocols

Primer and M13 Tag Sets				
	Blue	**Green**	**Yellow**	**Red**
M 13 Tag	FAM®	VIC®	PET®	NED®
Primer 1	SW 240	SW24	SW122	SO178
Primer 2	SO155	SO228	SW2406	SW632
Primer 3	SO226	SW857	SO002	SO101
Primer 4	SO090	SW830		

2. Run PCR Program as follows

1. 95° C for 5:00 minutes
2. 94° C for 1:00 minutes
3. 58° C for 1:30 minutes
4. 72° C for 1:30 minutes
5. 34 xs to Step 2
6. 72° C for 60:00 minutes
7. 10° C for 5:00 minutes
8. END

Post-PCR Processing:

1. Combine 3 µL of Blue, Green, and Yellow PCR Product with 5 µL of Red PCR Product.
2. Elute with 75 µL Optima H_2O.
3. Aliquot 4 µL of eluted PCR product into Promega WizardR SV96 ® binding plates.
4. Submit to Keck Center for Sequencing.

Appendix E: Effect of independent laboratory assessment, freezing volume, and other factors influencing post-thaw quality of frozen boar sperm

Abstract for Oral Presentation at the 2011 American Society of Animal Sciences National Meeting, New Orleans, LA

J.M. Ringwelski and R.V. Knox, 2011.

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Consistent and accurate post-thaw quality evaluation is essential in order to ensure proper usage of frozen thawed boar sperm (FTS) for artificial insemination. However, there are numerous sources of variation that contribute to inconsistent post-thaw quality measures. Laboratory Assessment which is classified as the as evaluation methods and procedures, is one of these sources of variation. Semen evaluation is traditionally done using either a computer assisted semen analysis (CASA) system, or through manual, subjective, evaluation for motility concentration and viability. Discrepancies between systems may account for significant variation in post thaw quality measures. Freeze volume is also often attributed to post thaw quality variation in FTS. The most common freeze volumes for boar semen are 0.5 mL and 5 mL. However, extensive research in semen packaging systems has indicated no significant effect in post thaw quality attributed to freeze volume. Finally, other effects that are often cited for post thaw variation include the boar's individual ability to freeze, breed effects, and seasonal effect. Frozen boar sperm shows lower fertility compared to liquid semen, which has been partly classified based on post-thaw lab assessments. Experiment 1 was performed to evaluate effects of independent lab assessment of post-thaw motility following freezing in 5 mL (Lab 1) or 0.5 mL (Lab 2) straws. Ejaculates (n = 117) from 27 mature boars of Landrace (n = 5), Large White (n = 15), Duroc (n = 5), and "Other" (n = 2) breeds were collected and frozen across seasons (winter-summer) from Feb to Jun 2010. Ejaculates were collected, diluted 1:1 in Modena, and held at 17°C until processing (Lab 1) or upon arrival the next day (Lab 2). All samples were frozen within 24 h of collection. Once frozen, straws were stored at -196°C until analysis. Straws were thawed at 50° C for 45 s for 5 mL straws and 20 s for 0.5 mL straws and evaluated at 37°C upon thawing. Data were analyzed using SAS for the effects of lab and volume, breed and season. There was no effect ($P > 0.05$) of lab and volume (47.4 vs. 49.8%), breed or season on motility. Experiment 2 was conducted to determine the effect of independent lab on pre-freeze concentration and motility, and also effects of breed, season, and collection number on post thaw quality measures in 0.5 mL straws. Straws (n = 47) from 26 boars were thawed and evaluated for motility and membrane integrity using propidium iodide. Data were analyzed in SAS for effects of lab assessments on pre-freeze motility, concentration, and total sperm cells. Effect of breed, season, and collection number on motility and viability were also evaluated in 0.5 mL straws. Measures of concentration (0.82), motility (0.78), and total cells (0.67) were all positively related for independent lab assessments ($P < 0.001$). Motility

Appendix E (cont.):

(47%) and membrane integrity (51%) were not affected by breed, season, or collection number ($P > 0.05$). The results of these experiments suggest that independent lab assessments post-thaw can be highly related, and there is no significant difference related to freezing in 5 or 0.5 mL straws. In boars in active collection rotations, breed and season had no impact on post-thaw quality of frozen boar sperm.

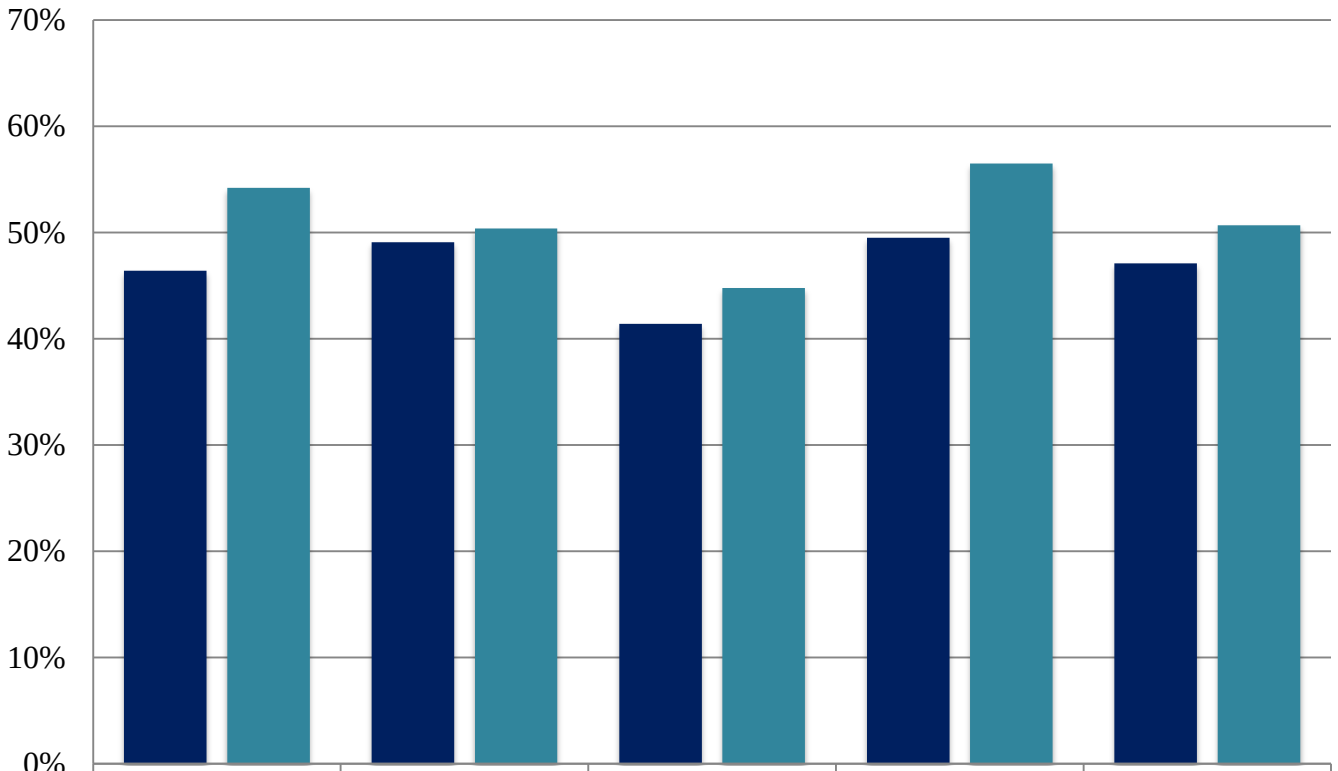
Appendix E (cont.).

Table E.1: Least square means of post-thaw motility in samples frozen in 5 mL (Treatment 1) and 0.5 mL (Treatment 2) straws. Data were analyzed using Mixed Procedures of SAS with main effects of laboratory and freeze volume.

Lab/ Treatment	**Freeze Volume**	**n**	**Post Thaw Motility**	**Range**	**P- Value**
1	5 mL	70	49.8 % ± 2.83	31-75%	$p > 0.05$
2	0.5 mL	47	47.4% ± 1.84	20-62%	$p > 0.05$

Appendix E (cont.).

Figure E.1: Least square means of the effect of breed on motility and membrane integrity estimates of the ejaculates frozen in 0.5 mL straws (n = 47).



	Landrace	Yorkshire	Duroc	Exotic	Total
Motility	46.4%	49.1%	41.4%	49.5%	47.1%
Membrane Integrity	54.2%	50.4%	44.8%	56.5%	50.7%